ABSTRACT

Title of Document: SUPPRESSORS OF *etr1-2*:
I. *etr1-11* IS A LOSS-OF-FUNCTION MUTATION OF THE *ETR1* ETHYLENE RECEPTOR.
II: *REVERSION TO ETHYLENE-SENSITIVITY3* IS A REGULATOR OF SEEDLING GROWTH

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The plant hormone ethylene is an important regulator of plant growth and development, including senescence, abcission, fruit ripening, and responses to biotic and abiotic stresses. To find new members of the ethylene signaling pathway, a genetic screen for suppressors of the ethylene-insensitive mutant *etr1-2* was performed. One mutant identified in this screen, *etr1-11*, is an intragenic mutation within *ETR1*. *etr1-11* is a unique missense mutation that appears to eliminate ETR1-2 signaling. Mutant analysis further revealed that *etr1-11* is a partial loss-of-function allele.

The *rte3* (*reversion to ethylene sensitivity3*) mutant was another mutant isolated in a genetic screen for suppressors of *etr1-2*. After testing other ethylene responses, such as leaf senescence, and performing epistasis analysis with other

ethylene signaling mutants, it was determined that *RTE3* is unlikely to play a direct role in the ethylene signaling pathway. Instead, *RTE3* appears to be responsible for promoting hypocotyl elongation in etiolated seedlings in the ethylene triple response assay.

The *RTE3* gene was identified by positional cloning, and is predicted to encode a protein with an annotated SAC3/GANP domain. SAC3/GANP domains are present in proteins that participate in large multi-peptide complexes, such as the 26S proteasome regulatory subunit and the eIF3 translation initiation complex. Similarities in protein composition between these two complexes and the COP9 signalosome (CSN) suggest that a SAC3/GANP domain-containing protein may interact with members of the CSN. Interestingly, yeast two-hybrid analysis reveals that RTE3 interacts with EER5 and EIN2, proteins that have been shown to interact with members of the CSN. In addition, *rte3-1 ein2-1* seedlings show a synthetic phenotype of delayed growth. Protein localization using a GFP tag reveals that RTE3 and EER5 both localize to the nucleus. These interactions suggest that RTE3, EER5, EIN2, and the CSN form a protein complex that regulates seedling growth.

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By

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Dissertation submitted to the Faculty of the Graduate School of the
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Dedication

When thinking back on the help needed to get my education, I can't help but think of the sheer number of people that assisted me in my education. To my parents, who were always supportive of an education and made many sacrifices to see that I received one. To all the teachers who helped me through school and truly loved what they did. To all of the college professors who spent a lot of time teaching well, when they could have been working on research. To the members of the CBMG department, who are always willing to assist a confused graduate student with the new equipment or the form that needs to be completed. To the lab members, who created a fun and productive atmosphere to work in, and who would always help me with any problem. I can't imagine earning a Ph.D. in any other lab. To my advisor, Caren, who was always there for assistance with any topic. She was always there with an answer to the latest research question, to make sure my papers and applications were top-notch, and just to talk. Thank you everybody.

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List of Abbreviations

2,4-D - 2,4-dichlorophenoxyacetic acid
3-AT - 3-amino-1,2,4-triazole
ABRC - *Arabidopsis* Biological Resource Center
ACC - 1-aminocyclopropane-1-carboxylic acid
ACS - ACC synthase
ASK - *Arabidopsis* Skp1-like
AVG - 2-aminoethoxyvinylglycine
AXR - auxin resistant
BAC - bacteria artificial chromosome
BLAST - basic local alignment search tool
BTB - Broad complex, Tamtrack, and Bric-a-brac
CAND - Cullin-associated, NEDD8-dissociated
CDC - cell division cycle
CDK - cyclin-dependent kinase
cDNA - complementary DNA
Col-0 - Columbia
COP - constitutive photomorphogenesis
CRL - Cullin-RING ligase
CSN - COP9 signalosome
CTR - constitutive triple response
CUL - Cullin
CYC - cyclin
DAPI - 4',6-diamidino-2-phenylindole
DNA - deoxyribonucleic acid
EBD - ethylene binding domain
EBF - EIN3-binding F-box
ECR - E1 C-terminal related
EER - enhanced ethylene response
EFH - EF hand
eIF - eukaryotic initiation factor
EIL - EIN3-like
EIN - ethylene insensitive
EMS - ethyl methanesulfonate
EOL - ETO1-like
ER - endoplasmic reticulum
ERF - ethylene response factor
ERS - ethylene response sensor
ETO - ethylene overproducer
ETP - EIN2-targeting protein
ETR - ethylene response
GAF - cGMP diesterase/adenylate cyclase/FhlA
GANP - germinal center associated nuclear protein
GAPC - glyceraldehyde-3-phosphate dehydrogenase C
GFP - green fluorescent protein

His - histidine
HY - elongated hypocotyl
Ler - Landsberg erecta
Leu - leucine
MAP - mitogen activated protein
MAPKKK - MAP kinase kinase kinase
MKK - MAP kinase kinase
MPK - MAP kinase
MPN - MPR1-PAD1-N-terminal
MS - Murashige and Skoog
NEDD - neural precursor cell expressed, developmentally downregulated
NR - never ripe
PAM - PCI-associated module
PARP - poly(ADP) ribose polymerase
PCI - proteasome/COP9/initiation factor
PCR - polymerase chain reaction
RBX - RING box
RCE - RUB-conjugating enzyme
RING - really interesting new gene
RNA - ribonucleic acid
RPN - regulatory particle non-ATPase
qRT-PCR - quantitative RT-PCR
RT-PCR - reverse transcriptase PCR
RTE - reversion to ethylene sensitivity
RUB - related to ubiquitin
SAC - suppressor of actin
SAM - S-adenosylmethionine
SCF - Skp-Cullin-F-box
SNP - single nucleotide polymorphism
SSLP - simple sequence length polymorphism
TAIR - The *Arabidopsis* Information Resource
TB - terrific broth
Ub - ubiquitin
UTR - untranslated region
Ws - Wassilewskija
XRN - exoribonuclease

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Chapter 1: Introduction

Ethylene Biosynthesis

Unlike animals, plants are non-motile organisms that cannot evade challenges presented to them. Plants must respond to the stresses they encounter. Plants respond to these stresses by utilizing a complex system of signaling pathways that respond to endogenously produced hormones. In addition to helping a plant respond to both biotic and abiotic stresses, these hormones also control the proper spatial and temporal development of plant tissues and organs. One of these hormones, ethylene, is a simple hydrocarbon that is a gas under physiological conditions. Ethylene has been shown to control a diverse set of plant processes, including fruit ripening, senescence, abscission, cell growth, and responses to both biotic and abiotic stresses (Abeles et al., 1992).

Ethylene has been known to be a plant hormone since 1901 when Neljubov found that the active compound within illuminating gas, used at the time to power street lamps, was ethylene. Illuminating gas had been known to trigger leaf abscission in trees, and Neljubov also found that pea seedlings would grow differentially in illuminating gas (Bleecker and Kende, 2000). In addition, it was later shown that plants produce ethylene gas; further supporting that ethylene is a plant hormone (Gane, 1934). In plants, ethylene is synthesized via a two-step process from S-adenosylmethionine (SAM). In the first, rate-limiting step, SAM is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS). ACC oxidase then converts ACC into ethylene (Bleecker and Kende, 2000). In addition to producing ACC, ACS also makes 5'-methylthioadenosine, which is recycled to

regenerate methionine, and is now known as the Yang cycle (Miyazaki and Yang, 1987; Wang et al., 2002).

The ACS enzymes require a pyridoxal phosphate cofactor, and are encoded by a multigene family that is differentially regulated in all plant species (Yu et al., 1979; Wang et al., 2002). In the model plant *Arabidopsis thaliana*, there are eight functional ACS enzymes, which are upregulated in response to ethylene (Yamagami et al., 2003; Liang et al., 1992). However, transcriptional regulation is a relatively slow process, and to quickly produce ethylene in response to stress, plants must have a faster response. A mechanism by which ethylene synthesis is upregulated in a rapid manner has been revealed through the *ethylene overproducer* (*eto*) mutants of *Arabidopsis*. *eto1*, *eto2*, and *eto3* have a constitutive ethylene response phenotype due to the overproduction of ethylene (Guzman and Ecker, 1990; Kieber et al., 1993).

The dominant *eto2* and *eto3* mutants are the result of mutations within the C-terminal ends of ACS5 and ACS9, respectively. These mutations increase the stability of these enzymes, suggesting that the ACS proteins are regulated through protein degradation (Vogel et al., 1998; Chae et al., 2003). This hypothesis was confirmed through the study of the *eto1* mutant. *ETO1* and its two homologs, *ETO1-LIKE1* and *ETO1-LIKE2* (*EOL1* and *EOL2*), encode BTB-motif E3 ligase proteins that interact with the C-termini of certain ACS proteins to target them for ubiquitin-mediated protein degradation (Wang et al., 2004; Yoshida et al., 2005; Christians et al., 2009). The ability to rapidly change the production rate of ethylene allows the plant to respond quickly to an external stimulus. However, said plant must still be able to sense this rapid flux in ethylene production to have a meaningful response.

The Ethylene Receptors

The mechanism by which plants sense ethylene has been largely elucidated by the use of mutant screens in *Arabidopsis thaliana*. These mutant screens have largely taken advantage of the triple response, a phenotype exhibited by etiolated seedlings when exposed to ethylene. The phenotype was initially described in pea seedlings, and is characterized by an exaggerated apical hook, a shortened and thickened hypocotyl, and a shortened root with a proliferation of root hairs (Crocker, 1932). By screening for mutant seedlings that do not undergo the triple response when ethylene is present, or for seedlings that undergo the triple response when no ethylene is present, many of the components of the ethylene signaling pathway have been discovered.

The first mutant discovered by screening a mutagenized population for ethylene insensitive mutants was the *ethylene response1* (*etr1*) mutant, a dominant mutation (Bleecker et al., 1988). Through the process of chromosome walking, the *ETR1* gene was cloned. The *ETR1* gene codes for a protein that shows homology to proteins of the two-component system in prokaryotes (Chang et al., 1993). In the two-component system, a sensor kinase detects a stimulus, which leads to autophosphorylation on a histidine residue. This phosphate is then transferred to a receiver domain on a response regulator protein. This transfer leads to an output signal by the response regulator (Parkinson and Kofoid, 1992). Interestingly, ETR1 contains both a histidine kinase and a receiver domain, suggesting that ETR1 could perform both sensor and output functions of the two-component system.

ETR1 also contains an N-terminal hydrophobic domain that was later shown to be capable of binding ethylene (Schaller and Bleecker, 1995). This region also contains two cysteine residues, which are required for the dimerization of ETR1 (Schaller et al., 1995). In addition, the ETR1 protein requires a copper ion cofactor to bind ethylene, and loss-of-function mutations in the copper transporter gene *RAN1* lead to strong constitutive ethylene responses (Rodriguez et al., 1999; Hirayama et al., 1999; Woeste and Kieber., 2000). Within the ethylene binding domain (EBD) lie all known ethylene-insensitive mutations in *ETR1*. A site-directed mutagenesis approach using *etr1* transgenes has revealed three classes of mutations within the EBD. One class of mutations abolishes ethylene binding, another retains ethylene binding but fails to transmit the 'turn off' signal to downstream components, and a final class is loss-of-function mutants (Wang et al., 2006). ETR1 also contains a c**G**MP diesterase/**a**denylate cyclase/**F**hlA (GAF) domain, which is involved in protein-protein interactions among the ethylene receptors (Gao et al., 2008).

The ETR1 ethylene receptor is a member of a family of five ethylene receptors, which are negative regulators of ethylene signaling (Hua and Meyerowitz, 1998). The other ethylene receptor genes, *ERS1*, *ETR2*, *EIN4*, and *ERS2*, were found through either sequence homology with *ETR1* or through screens for ethylene insensitive mutants (Hua et al., 1995; Hua et al., 1998; Sakai et al., 1998). The ethylene receptors can be divided into two subfamilies based on sequence similarity. Subfamily I, consisting of ETR1 and ERS1, contains all residues necessary for histidine kinase activity and have three transmembrane domains at the N-terminus.

Subfamily II, consisting of ETR2, EIN4, and ERS2, contain degenerate histidine kinase motifs and four N-terminal transmembrane domains (Hua et al., 1998).

Due to the redundancy of the ethylene receptors, loss-of-function mutants of any single receptor do not result in strong phenotypes. Loss-of-function mutations in *ETR1* and *ERS1* result only in a slight ethylene hypersensitivity, while single loss-of-function mutants for the subfamily II receptors result in no phenotype (Hua and Meyerowitz, 1998; Cancel and Larsen, 2002; Qu et al., 2007). Multiple ethylene receptor knockout mutants lead to increasingly stronger constitutive ethylene-response phenotypes (Hua and Meyerowitz, 1998). Subfamily I receptors seem to play a stronger role in ethylene response and plant development as a mutant null for both subfamily I receptors leads to a much stronger constitutive response phenotype as well as failure to produce seeds (Hall and Bleecker, 2003; Qu et al., 2007).

While the subfamily I ethylene receptors ETR1 and ERS1 play a more prominent role in ethylene signaling, it is not due to their conserved histidine kinase residues. *In vitro* studies have shown that both ETR1 and ERS1 have histidine kinase activity, but ERS1 histidine kinase activity disappears in the presence of magnesium, similar to physiological conditions (Gamble et al., 1998; Moussatche and Klee, 2004). In addition, an *etr1* transgene, defective in its histidine kinase activity, is still able to rescue a multiple ethylene receptor knockout, suggesting that histidine kinase activity is not required for ethylene receptor function (Wang et al., 2003). However, the histidine kinase domain is still necessary for receptor function, perhaps suggesting that this domain has evolved a new function to participate in ethylene signaling (Qu and Schaller, 2004). Indeed, all ethylene receptors in *Arabidopsis*, excluding ETR1,

show serine/threonine kinase activity (Moussatche and Klee, 2004). Since this activity is not present in ETR1, this activity is unlikely to be functional in canonical ethylene signaling. Whether serine/threonine kinase activity is important in ERS1, ETR2, EIN4, and ERS2 function remains unclear.

The prominence of *ETR1* in ethylene signaling has been revealed through the studies of its interaction with a novel gene, *REVERSION-TO-ETHYLENE SENSITIVITY1* (*RTE1*). *RTE1* was initially identified as a loss-of-function mutant that suppressed the ethylene insensitivity of the *etr1-2* mutant and is required for *ETR1* function. Although there is no known biochemical function for *RTE1*, some idea of the function of *RTE1* has been gained through its genetic interactions. *rte1* loss-of-function mutants are only able to suppress ethylene-insensitive mutations within *ETR1* and not within any of the other *Arabidopsis* ethylene receptor genes (Resnick et al., 2006). Furthermore, only a subset of the dominant, ethylene-insensitive mutations within *ETR1* is suppressed by *rte1* (Resnick et al., 2008). These data have also shown that *ETR1* is unique among the *Arabidopsis* ethylene receptors, as these *RTE1*-dependent ethylene-insensitive mutations within *ETR1* are not transferable to other ethylene receptors, while *RTE1*-independent mutations are able to confer ethylene insensitivity upon the other receptors in *Arabidopsis* (Rivarola et al., 2009).

The genetic interaction between *RTE1* and *ETR1* has been supported by the co-localization of the two proteins (Dong et al., 2008). ETR1 had been localized to the endoplasmic reticulum (ER) through sucrose gradient fractionation, but fluorescent protein tag experiments show that both RTE1 and ETR1 localize to both

the ER and the Golgi apparatus (Chen et al., 2002; Zhou et al., 2007; Dong et al., 2008). In addition to *ETR1* regulation through *RTE1*, other ethylene receptors may be regulated through protein degradation. Studies in *Arabidopsis* have shown that the ETR2 ethylene receptor is degraded upon treatment with ethylene and the tomato ethylene receptors LeETR4 and LeETR6 are likewise degraded with ethylene treatment (Chen et al., 2007; Kevany et al., 2007). Whether any of the other ethylene receptors in *Arabidopsis* are degraded in response to ethylene remains unknown.

Downstream Ethylene Signaling

In addition to the ethylene receptors, several other components of the ethylene signaling pathway were isolated through genetic screens for both ethylene-insensitive and constitutive ethylene-response mutants (See Fig. 1). *CONSTITUTIVE TRIPLE RESPONSE1* (*CTR1*) was isolated as a recessive mutant which exhibited a constitutive ethylene response, even without ethylene treatment. *CTR1* is a negative regulator of ethylene signaling and encodes a Raf-like kinase with a novel N-terminal domain (Kieber et al., 1993). *CTR1* acts immediately downstream of the receptors in the ethylene signaling pathway. In fact, it was shown that CTR1 physically interacts with both ETR1 and ERS1 (Clark et al., 1998). Furthermore, CTR1 was localized to the ER, and this localization is dependent upon the presence of the ethylene receptors (Gao et al., 2003).

CTR1 shows homology to the MAPKKK Raf in mammalian systems through its kinase domain and is an active serine/threonine kinase (Huang et al., 2003). The presence of a MAPKKK homolog in the ethylene signaling pathway has

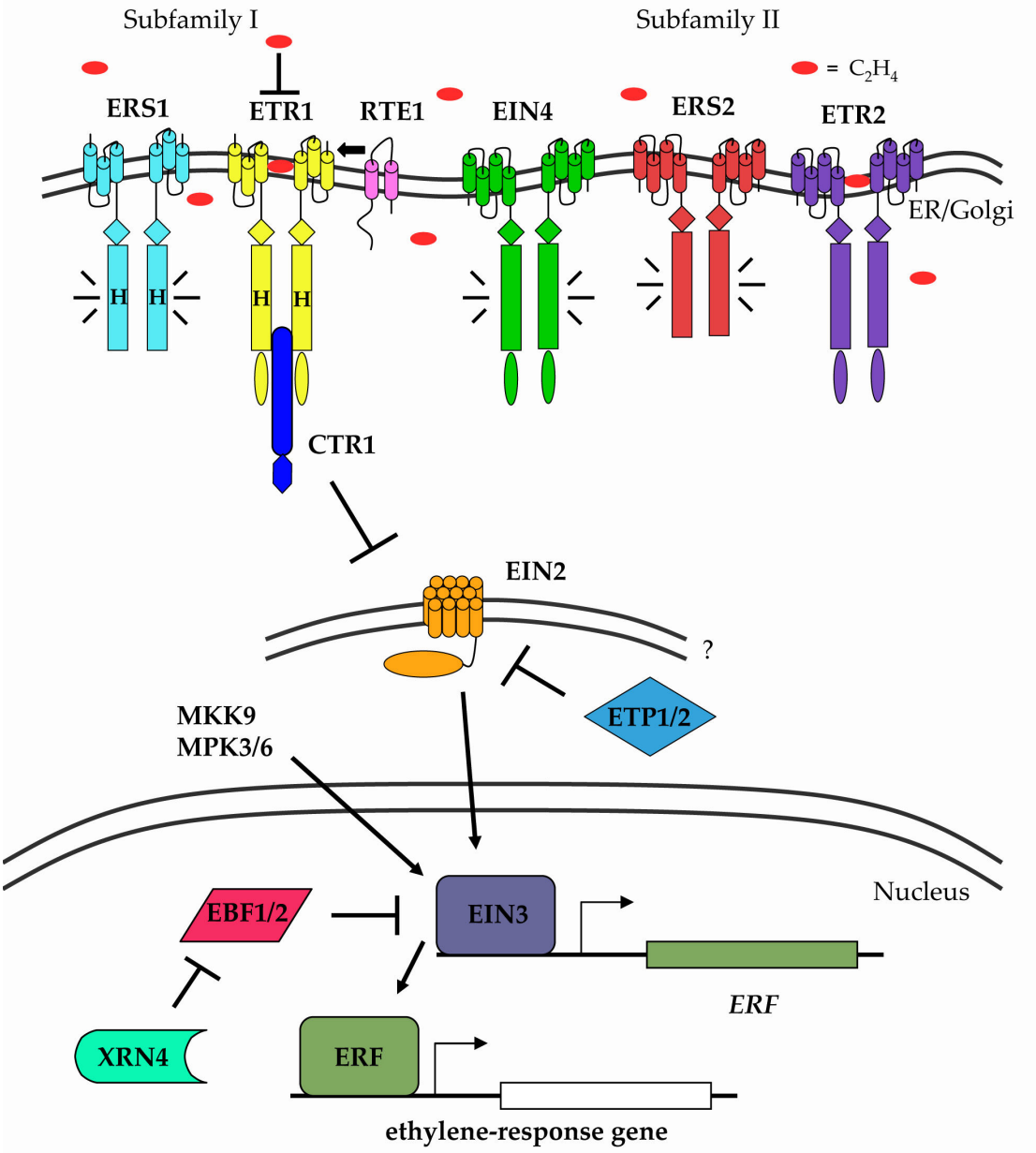

Subfamily I
Subfamily II
= C_2H_4
ERS1
ETR1
RTE1
EIN4
ERS2
ETR2
ER/Golgi
H
H
H
H
CTR1
EIN2
?
ETP1/2
MKK9
MPK3/6
Nucleus
EBF1/2
EIN3
ERF
XRN4
ERF
ethylene-response gene

Figure 1 - Overview of the ethylene signaling pathway. In the absence of ethylene, the five receptors actively repress responses through the Raf-like kinase CTR1. When ethylene binds, the receptors and CTR1 are turned off, allowing responses to occur. The protein RTE1 specifically keeps the ETR1 ethylene receptor active in the absence of ethylene. Downstream of the receptors and CTR1, EIN2 is a positive regulator of responses and activates the transcription factor EIN3. The F-box proteins ETP1/2 repress EIN2 protein levels in the absence of ethylene. The F-box proteins EBF1/2 repress EIN3 protein levels in the absence of ethylene. XRN4 negatively regulates EBF1/2 levels through an unknown mechanism. EIN3 induces expression of the transcription factor ERF, which activates expression of ethylene response genes. ETR1/2 = ETHYLENE RESISTANT1/2; ERS1/2 = ETHYLENE RESPONSE SENSOR1/2; EIN2/3/4 = ETHYLENE INSENSITIVE 2/3/4; CTR1 = CONSTITUTIVE TRIPLE RESPONSE1; ETP1/2 = EIN2 TARGETING PROTEIN 1/2; EBF1/2 = EIN3 BINDING F-BOX1/2; XRN4 = EXORIBONUCLEASE4; ERF = ETHYLENE RESPONSE FACTOR; MKK = MAP KINASE KINASE; MPK = MAP KINASE

led to the speculation that a MAP kinase module may be involved in ethylene signaling. It was shown that, while not an entirely linear pathway from CTR1 to downstream signaling components, a MAP kinase module consisting of MKK9 and MAPK3/6 participates in ethylene signaling (Yoo et al., 2008). Another group has shown that the MKK9/MAPK3/6 module stimulates ethylene synthesis, however (Xu et al., 2008). Whether this MAP kinase module participates in ethylene signaling, ethylene biosynthesis, or both, is yet to be determined.

Another component of the ethylene signaling pathway is *ETHYLENE INSENSITIVE3* (*EIN3*), isolated as a recessive, ethylene-insensitive mutant. *EIN3* is a transcriptional activator of ethylene responses, through binding the primary ethylene response element (PERE) of the *ETHYLENE RESPONSE FACTOR* (*ERF*) gene (Chao et al., 1997; Solano et al., 1998). ERF is itself a transcription factor and drives expression of ethylene response genes by binding to the ethylene response element (ERE) within their promoters (Solano et al., 1998). *EIN3* is a member of a family of transcription factors in *Arabidopsis* that includes five *EIN3-LIKE* (*EIL*) genes. Only *EIL1* and *EIL2* have been shown to rescue *ein3* loss-of-function mutants (Chao et al., 1997).

Although the downstream events in the activation of ethylene response genes had been characterized, the question of how the EIN3 protein is activated remained unclear. A trio of papers resolved this question, when it was revealed that the F-box proteins EIN3-BINDING F-BOX1 and 2 (EBF1 and EBF2) are members of the ethylene signaling pathway (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004). EBF1 and EBF2 target EIN3 for degradation, and *ein3* or *eil1* mutants can

rescue the constitutive ethylene phenotype exhibited by *ebf1 ebf2* double mutants (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004; Binder et al., 2007). The fact that there is a multiple gene family consisting of *EIN3* and *EIL* genes, as well as two *EBF* genes suggests there may be distinct functions for these proteins. Indeed, the inhibition of hypocotyl growth after ethylene treatment shows a biphasic pattern. The first, rapid response to ethylene is comparatively weak, but is independent of both EIN3 and EIL1. The second, slower response to ethylene is stronger, but dependent upon EIN3/EIL1 (Binder et al., 2004). Furthermore, the roles of EBF1 and EBF2 in the regulation of this biphasic response seem to be different. *ebf1* mutants initiate the second stage of hypocotyl growth inhibition faster, while *ebf2* mutants are delayed in recovery after removal of ethylene (Binder et al., 2007). Additionally, the *EBF2* promoter, but not the *EBF1* promoter, is activated by EIN3, suggesting a feedback mechanism for ethylene responses (Konishi and Yanagisawa, 2008).

A key question that remains is how the EBF F-box proteins are regulated. Recent findings indicate that the exoribonuclease XRN4 regulates *EBF1* and *EBF2* transcript levels. The *EIN5* locus, which was first identified in a screen for ethylene-insensitive *Arabidopsis* mutants, was found to encode XRN4, and epistasis analysis showed that *EIN5/XRN4* lies downstream of *CTR1* but upstream of *EBF1* and *EBF2* (Olmedo et al., 2006; Potuschak et al., 2006). However, *EBF* transcript turnover rate is the same between wild type and *ein5/xrn4* mutants, indicating that XRN4 fails to directly degrade *EBF* transcripts (Potuschak et al., 2006).

Another component of the ethylene signaling pathway isolated through a genetic screen is *ETHYLENE INSENSITIVE2* (*EIN2*). Isolated as a recessive, ethylene-insensitive mutant, *EIN2* encodes a membrane protein that has no known molecular function, but has homology to N-ramp metal transporter proteins (Alonso et al., 1999). *EIN2* lies downstream of *CTR1* and upstream of *EIN3* in the ethylene signaling pathway, and expression of the EIN2 C-terminus is sufficient to activate ethylene responses (Alonso et al., 1999). Although no molecular function is known for the EIN2 protein, a method for its regulation has been found. EIN2 is also targeted for degradation by two F-box proteins, EIN2 TARGETING PROTEIN1 and EIN2 TARGETING PROTEIN2 (ETP1 and ETP2) (Qiao et al., 2009).

While a mechanism for EIN2 regulation has been discovered by the interaction between EIN2 and ETP1/2, the manner in which EIN2 exerts its effects on downstream components remains obscured. A clue may be given by a recent study in which it was found that the C-terminus of EIN2 interacts with the **P**roteasome/**C**OP9/**I**nitiation factor (PCI) domain-containing protein ENHANCED ETHYLENE RESPONSE5 (EER5) (Christians et al., 2008). The PCI domain is a presumed protein-interaction domain present within many proteins that are members of multiple protein complexes like the 26S proteasome regulatory subunit, the COP9 signalosome (CSN), and the eukaryotic translation initiation factor3 (eIF3) complex (Hofmann and Bucher, 1998). This led to the finding that EIN2 interacts with two members of the CSN as well as EER5 (Christians et al., 2008). While the interaction with the CSN does not solve the question of how EIN2 exerts its effects on

downstream signaling components, it may lead to a molecular function for the protein.

The COP9 Signalosome

The CSN is a multi-protein complex consisting of eight subunits (CSN1 to CSN8). The original members of the CSN were identified through genetic screens for mutants that displayed constitutive photomorphogenic phenotypes (Wei et al., 2008). Although initially identified in *Arabidopsis*, the CSN is present in all eukaryotes. In most organisms, the CSN is a complex of eight proteins; six contain the aforementioned PCI domain, while the other two contain an **M**PR1-**P**AD1-**N**-terminal (MPN) domain (Wei and Deng, 2003). The best understood biochemical function for the CSN is the cleavage of the ubiquitin-like protein RUB1 (plants)/NEDD8 (animals and yeast) from the CULLIN (CUL) proteins, which are central members of the protein degradation pathway in eukaryotes (Lyapina et al., 2001; Gusmaroli et al., 2007).

RUB1 is a key regulator of the CULLIN-RING ligase (CRL) system of protein regulation in eukaryotes. The CRL protein complex performs the final step in the conjugation of ubiquitin (Ub) to a target protein. Once a protein has been ubiquitinated, it is targeted to the 26S proteasome for degradation (Hotton and Callis, 2008). The ubiquitin molecule is activated for attachment to target proteins by ubiquitin activating enzymes, or E1 proteins. E1 enzymes activate the ubiquitin molecule by adenylating the ubiquitin peptide, then forming a covalent linkage with ubiquitin. There are only two E1 proteins present in the *Arabidopsis* genome, suggesting that control of substrate specificity is not conferred by the E1 enzymes

(Hatfield et al., 1997). After activation, the E1 enzymes transfer the ubiquitin moiety to a ubiquitin conjugating enzyme, or E2 enzyme. It is from the E2 protein that the ubiquitin moiety is transferred to the target protein. It is the function of the ubiquitin ligases, or E3 proteins, to perform the transfer reaction. E3 ligases interact with target proteins to bring the targets to the E2-ubiquitin conjugate and to catalyze the transfer of ubiquitin to the target proteins. The E3 ligases are a very diverse group of proteins, as there are over 1300 genes encoding E3 ligase subunits in *Arabidopsis* (Vierstra, 2003).

There are several classes of E3 ligases, but the most diverse set of E3 enzymes are the CULLIN RING LIGASE (CRL) complexes. The CRL complex consists of a cullin protein that acts as a bridge between a RING protein that recognizes the E2 conjugating enzyme and the target recognition protein. There are several classes of CRL complexes, including the well-known Skp-Cullin-F-box (SCF) complex in which an *Arabidopsis* SKP1-like (ASK) protein bridges CULLIN1 (CUL1) and an F-box protein that acts as the target recognition protein (Hotton and Callis, 2008). More than 700 F-box proteins have been annotated in the *Arabidopsis* genome (Smalle and Vierstra, 2004). Coupled with the other types of CRL complexes, including the CUL3-**B**road-Complex, **T**amtrack, and **B**ric-a-Brac (BTB) type of CRL E3 ligases, there are thousands of possible modules to target proteins to the proteasome.

RUB1 modification (or rubylation) of the cullin proteins is a covalent modification similar to the ubiquitination process. RUB1 must be activated and conjugated, similar to ubiquitin, and the proteins AUXIN RESISTANT1 (AXR1) and E1 C-TERMINAL RELATED1 (ECR 1) act as a functional RUB1-activating (E1)

enzyme, while RUB-CONJUGATING ENZYME1 (RCE1) acts as a RUB1-conjugating (E2) enzyme in *Arabidopsis* (del Pozo and Estelle, 1999). Surprisingly, there is no E3 ligase complex analogous to the CRL for RUB1, but the RING protein RING BOX1 (RBX1), which is a component of the CRL ubiquitin ligase complex, is required for CUL1 rubylation (Kamura et al., 1999; Morimoto et al., 2003). The modification of the cullin proteins by RUB1 is an essential step for CRL function. Mutants that decrease the amount of activated RUB1 (and hence, rubylated cullins) accumulate proteins that are normally targeted for degradation and generally show altered auxin responses (del Pozo et al., 2002; Gray et al., 2001; Zenser et al., 2003). Additionally, knock-outs in the genes involved in the rubylation pathway are embryo lethal, suggesting that rubylation is required for the most basic developmental processes (Bostick et al., 2004; Dharmasiri et al., 2007; Hotton and Callis, 2008).

The CSN is important in the activity of the CRL complexes due to its removal of RUB1 from the cullin proteins (derubylation) in eukaryotes. The loss of any component of the CSN in *Arabidopsis* results in constitutive photomorphogenic and seedling lethal phenotypes (Wei and Deng, 2003; Gusmaroli et al., 2007). Consistent with its function as a derubylation complex, the *Arabidopsis* cullins are constitutively rubylated in *csn* mutants (Gusmaroli et al., 2007). However, partial loss-of-function mutants within the CSN have diminished auxin responses and accumulate SCF targets, similar to mutants within the rubylation pathway (Gusmaroli et al., 2004; Gusmaroli et al., 2007). *In vitro* studies have shown that the CSN inhibits target protein ubiquitination, contrary to *in vivo* analysis (Lyapina et al., 2001; Yang et al., 2002).

One hypothesis for the opposite effects seen in these studies is that CRL complexes must go through a cycle of rubylation/derubylation to be active. This is supported by the finding that when rubylated, CRL component proteins are themselves more likely to be ubiquitinated, leading to a decreased number of stabilized CRL complexes and more stabilized target proteins (Zhou and Howley, 1998; Galan and Peter, 1999). This has been supported by *in vivo* work that shows the cullin proteins are less abundant in *csn* mutants (Gusmaroli et al., 2007). This has led to the model through which cycles of rubylation and derubylation are required for proper CRL function, which has been supported through genetic analysis (Zhang et al., 2008).

Further support for the cycle model of CRL activity comes from the study of the protein **C**ULLIN-**A**SSOCIATED **N**EDD8-**D**ISSOCIATED1 (CAND1). In *Arabidopsis*, *cand1* mutants were identified to have defective responses to multiple plant hormones, including auxin. These responses mimicked those seen in CRL component mutants (Feng et al., 2004; Chuang et al., 2004; Cheng et al., 2004). Although *Arabidopsis cand1* mutants do not affect the rubylation status of the cullin proteins, they still accumulate CRL target proteins, suggesting that CRL function is compromised in these mutants (Feng et al., 2004; Chuang et al., 2004). The molecular function of CAND1 has been hypothesized to be a negative regulator of CRL ubiquitination activity, based on the finding that CAND1 prevents Skp1/F-box protein binding to CUL1 and vice versa (Zheng et al, 2002; Bornstein et al., 2006). These *in vitro* findings conflict with the *in vivo* data of the *cand1* mutants, which suggests that CAND1 is required for optimal CRL function and is similar to the CSN

mutant data. Combined, these data have led to the hypothesis that cycles of CRL rubylation and derubylation are required for optimal CRL function, summarized in Figure 2 (Hotton and Callis, 2008).

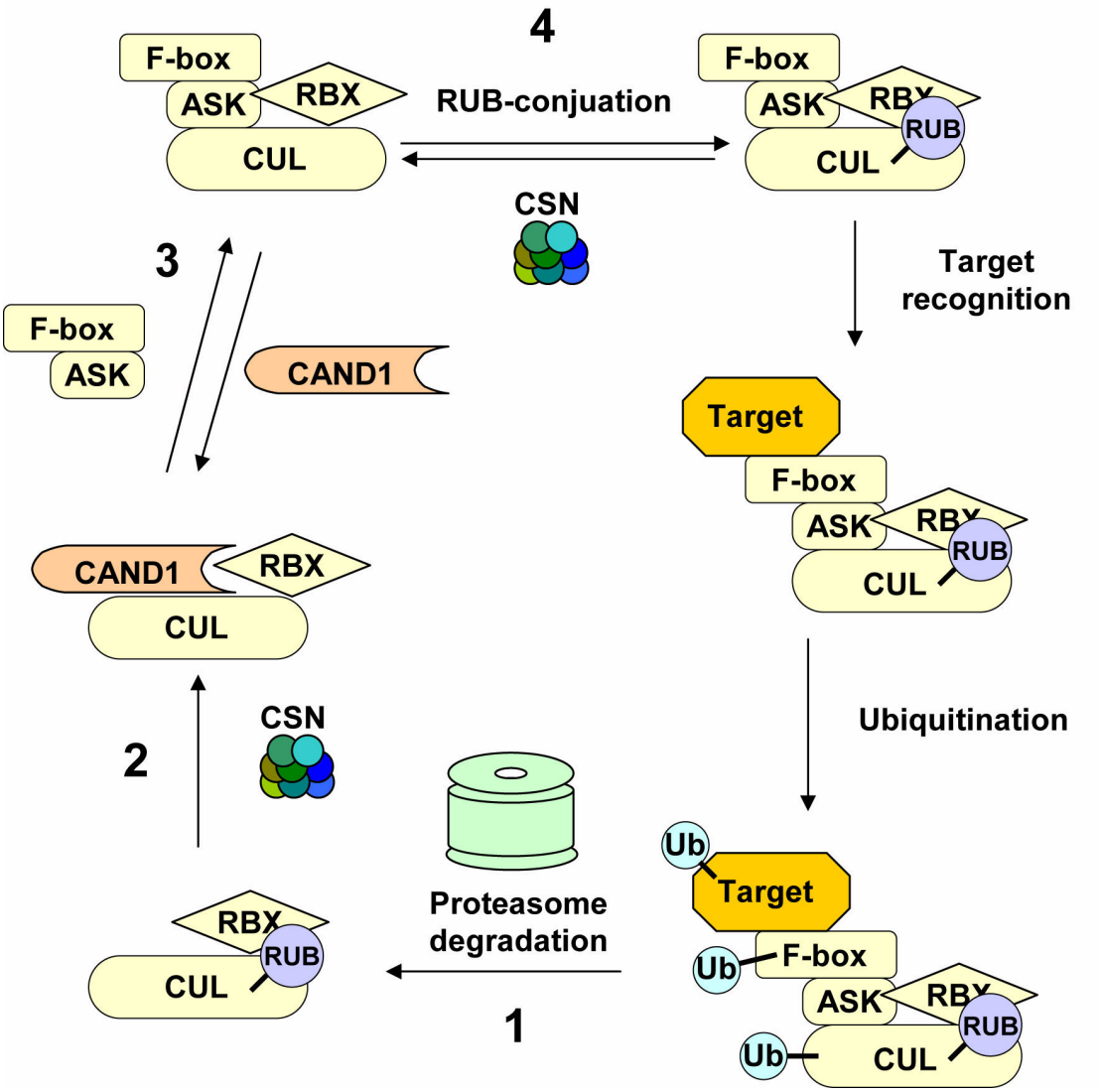


Figure 2 - The CRL complexes undergo a complex regulatory mechanism. RUB-conjugation of the Cullin (CUL) protein promotes active targeting of proteins for

degradation, as well as ubiquitination of the CRL proteins themselves. After target protein(s) have been degraded (1), the CSN removes the RUB protein, and allows CAND1 to bind the CUL protein (2). CAND1 dissociation promotes CRL complex formation (3), which is activated by a new cycle of rubylation (4). CUL = CULLIN; ASK = *Arabidopsis* SKP1-LIKE; RUB = RELATED TO UBIQUITIN; Ub = UBIQUITIN; RBX = RING BOX1; CAND1 = CULLIN ASSOCIATED NEDD8 DISSOCIATED1; CSN = COP9 signalosome. Adapted from Hotton and Callis (2008)

Despite the depth of knowledge gained about the rubylation/derubylation pathway, there are still many questions remaining. For instance, what are the functions of some of the CRL complexes. Although CUL2 has been implicated in cell cycle regulation in animal systems, no known physiological role for it has emerged in *Arabidopis* (Willems et al., 2004). Another mystery in the CSN field is the fact that CSN proteins can participate in protein complexes besides the CSN. What these complexes do or how they are regulated remains unknown (Gusmaroli et al., 2007).

Current Questions in Ethylene Signling

The ethylene receptors have been well characterized in genetic studies, yet the molecular mechanisms by which they signal to downstream components remains elusive. This may be due to genetic redundancy of some unknown, yet key, signaling components. It has been hypothesized that these may include MAP kinases, since CTR1 is a Raf-like MAPKKK, yet no direct evidence links CTR1 with any MAPKK or MAPK proteins. It is also unclear how the deactivation of the F-box proteins within the ethylene signaling pathway occurs. The EBF proteins regulate the levels of EIN3 in response to ethylene, and the ETP proteins function similarly towards EIN2. Yet it remains unknown how these proteins are deactivated in response to ethylene to allow these proteins accumulate. Also of interest is the molecular function of EIN2. Although loss-of-function mutants within *EIN2* are ethylene insensitive, the mechanism by which EIN2 exerts these effects remains unknown. Even the sub-cellular localization of the EIN2 protein is a mystery. The discovery of

these unknowns will go a long way towards a greater understanding of ethylene signaling.

Although no ethylene-related phenotypes have been reported for any mutants of the CRL-regulation apparatus, the recent work on *EER5* from Christians et al. (2008) indicates that the CSN may play a role in regulating the ethylene signaling pathway. This is a likely hypothesis considering that the F-box proteins EBF1 and EBF2 are members of a CRL complex and regulate the levels of the transcription factor EIN3. Therefore, any change in the activity of the CRL would likely have an effect on ethylene signaling. The question remains however, whether there is any specific regulation of the CSN, and hence, CRL activity from the ethylene signaling pathway itself.

Conclusion

Although much progress has been made within the ethylene signaling field, there remains even more to be discovered. From the receptors to gene regulation, there are questions at every level of the pathway to be answered. In this dissertation, I aim to contribute to the knowledge of how the ethylene signaling pathway is regulated through the use of a genetic screen to find suppressors of the ethylene-insensitive allele *etr1-2*. The knowledge gained through this study will help to elucidate the mechanism by which the ethylene pathway functions at multiple levels, which will allow for applications in agronomically important processes.

An Intragenic Suppressor of *etr1-2*: *etr1-11*

Introduction

The ethylene receptors have been the center of an intense effort to discover how they signal to downstream components in the signaling pathway. The transmembrane regions of the receptors are the site of ethylene binding; the portion of ETR1 that contains the transmembrane segments was shown to be responsible for binding ethylene, and mutations that disrupt ethylene binding are located within this region (Schaller and Bleecker, 1995; Chang et al., 1993; Wang et al., 2006). Initial ethylene receptor mutants, such as *etr1-1* and *etr1-2*, were isolated based on their ethylene-insensitive phenotype, and are dominant gain-of-function mutants (Bleecker et al., 1988; Chang et al., 1993). However, the mechanism by which signaling occurs and why signaling fails to be terminated in ethylene-insensitive mutants remains unknown. As an example, *RTE1* is required for wild type *ETR1* function, as *rte1* mutants mimic *etr1* loss-of-function mutants. However, *etr1-1 rte1 Arabidopsis* plants are ethylene-insensitive, indicating that ETR1 is in a constitutive signaling state despite the loss of *RTE1* (Resnick et al., 2006). This is a contrast to *etr1-2 rte1 Arabidopsis* plants, which have a similar phenotype to *etr1* loss-of-function mutants (Resnick et al., 2006). The dichotomy between the two ethylene-insensitive mutants *etr1-1* and *etr1-2* indicates that the mechanisms by which ethylene is perceived and receptor signaling is deactivated are complex.

In addition to the question of what is required for the termination of receptor signaling, little is known about how the ethylene receptors are maintained in an 'on' state in the absence of ethylene. It has been difficult to isolate loss-of-function

mutations within the ethylene receptor genes due to their functional redundancy. Loss-of-function mutations isolated so far were found through intragenic suppression of dominant, ethylene-insensitive mutations, and these mutations have all been either splice site or nonsense mutations, leading to truncated proteins and yielding little information about residues important for ethylene receptor function. In a recent study by Wang et al. (2006), many residues within the ethylene-binding domain of ETR1 were mutated and their effect on receptor function was analyzed. Surprisingly, most mutations had either no effect on the receptor or caused a gain-of-function phenotype, that is, the receptors failed to turn 'off' in the presence of ethylene. Only two of the 41 mutations analyzed yielded a loss-of-function phenotype, which may indicate that many more residues within the ethylene-binding domain are important for turning off the receptor rather than maintaining it in an active signaling state.

The lack of missense loss-of-function mutations within the ethylene receptor genes has led to a lack of understanding in how the ethylene receptors are signaling. In this study, I present the identification of an *etr1* loss-of-function allele that suppresses *etr1-2* signaling. This mutation, named *etr1-11*, which was obtained in a screen for suppressors of the ethylene-insensitive mutant *etr1-2*, is a loss-of-function allele.

Results

etr1-11 is a loss-of-function allele of the ethylene receptor *ETR1*

To gain further insight into ethylene signaling, a genetic screen for suppressors of the weak ethylene-insensitive mutant *etr1-2* was performed. The M_2 generation of an ethyl methanesulfonate (EMS)-mutagenized *etr1-2* population was

screened for the triple response phenotype in the presence of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC). The mutants obtained in this screen were tested to ascertain whether they were intragenic to the *etr1-2* allele by crossing the mutants to wild type. Due to the dominant nature of *etr1-2*, when a suppressor mutant is crossed to wild type, the resulting F_1 will display ethylene insensitivity if the mutant is extragenic, while the resulting F_1 will exhibit the suppressed phenotype if the mutant is intragenic. The suppressor mutations were determined to be dominant or recessive by examining the F_1 phenotype of a back-cross to the *etr1-2* allele. In addition to several extragenic mutants that were isolated in this screen (Dong et al., unpublished data), one intragenic suppressor of the *etr1-2* mutation was isolated. This mutant, designated as *etr1-11*, was analyzed by characterizing the triple response of the mutant in an ethylene dose response assay (Figure 3B). The *etr1-11* mutant was found to have a hypersensitive response to ethylene similar to *etr1* loss-of-function mutants as assayed by the triple response. This effect is readily observed when etiolated seedlings are treated with a low dose of ethylene (0.1 ppm) (Figure 3A). It was also observed that *etr1-11* seedlings are shorter than wild-type seedlings even when not treated with ethylene, another characteristic of an ethylene hypersensitive phenotype (Figure 3B).

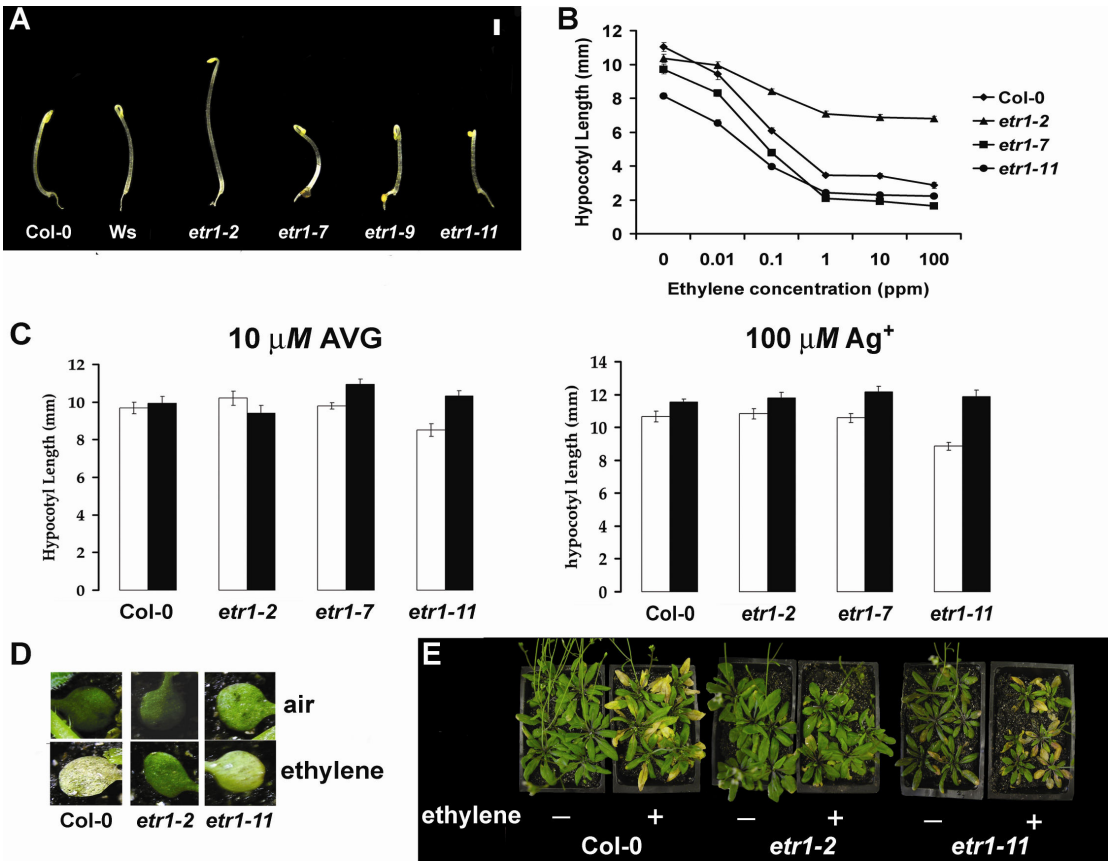


Figure 3 - The *etr1-11* mutant is hypersensitive to ethylene. A) Etiolated seedlings were grown on MS plates for four days in the presence of 0.1 ppm ethylene. Representative seedlings are shown. Scale bar is 1 mm. B) Dose response of *etr1-11* to ethylene. Etiolated seedlings were grown for four days on MS media in the presence of indicated amounts of ethylene gas. Data represents one experiment where n = 12 seedlings for each data point, error bars represent standard error of the mean. C) Etiolated seedlings were grown on either MS alone (white bars) or on 10 μ*M* AVG or 100 μ*M* silver nitrate (black bars). Data represents one experiment where n = 12 seedlings for each data point, error bars represent standard error of the mean. D) Plants were grown on soil for 17 days, then moved into airtight chambers

and treated with either air or 100 ppm ethylene. After three days of treatment, plants were removed and cotyledons photographed. E) Plants were grown for four weeks on soil, then moved to an airtight chamber and treated with either air or 100 ppm ethylene. After four days of treatment, plants were removed and rosettes were photographed.

This hypersensitive response is due to the small amount of ethylene produced by the seedling.

To test whether the reduced hypocotyl length exhibited by *etr1-11* seedlings not treated with ethylene was in response to endogenously produced ethylene or a general growth defect, *etr1-11* seedlings were treated with an inhibitor of ethylene synthesis, aminoethoxyvinylglycine (AVG), and an inhibitor of ethylene response, monovalent silver. When treated with either AVG or silver, *etr1-11* seedlings were restored to wild-type length, indicating that the *etr1-11* mutant phenotype is a result of enhanced ethylene response (Figure 3C). To assess whether the suppression phenotype of *etr1-11* was a whole plant response rather than seedling-specific, the senescence responses of the mutant were studied. Senescence was examined in the cotyledons of young *Arabidopsis* plants, which will senesce when treated with ethylene (Jing et al., 2002). When 17-day old *etr1-11* plants were treated with ethylene, their cotyledons senesced, providing further evidence that the *etr1-11* mutation suppresses the ethylene-insensitive *etr1-2* mutation (Figure 3D). Additionally, the *etr1-11* mutation was able to suppress the ethylene-insensitivity of *etr1-2* when assayed by leaf senescence in the rosettes of four-week old plants (Figure 3E). We therefore conclude that *etr1-11* is a loss-of-function mutation.

DNA sequencing revealed that the genetic lesion responsible for the *etr1-11* phenotype is a C to T transition in the *ETR1* nucleotide sequence. This missense mutation results in an arginine to tryptophan substitution at position 118 in the ETR1 protein sequence (Figure 4A). This is a novel mutation since it is the only missense mutation in the *ETR1* gene isolated in a mutagenic screen that is not a dominant gain-

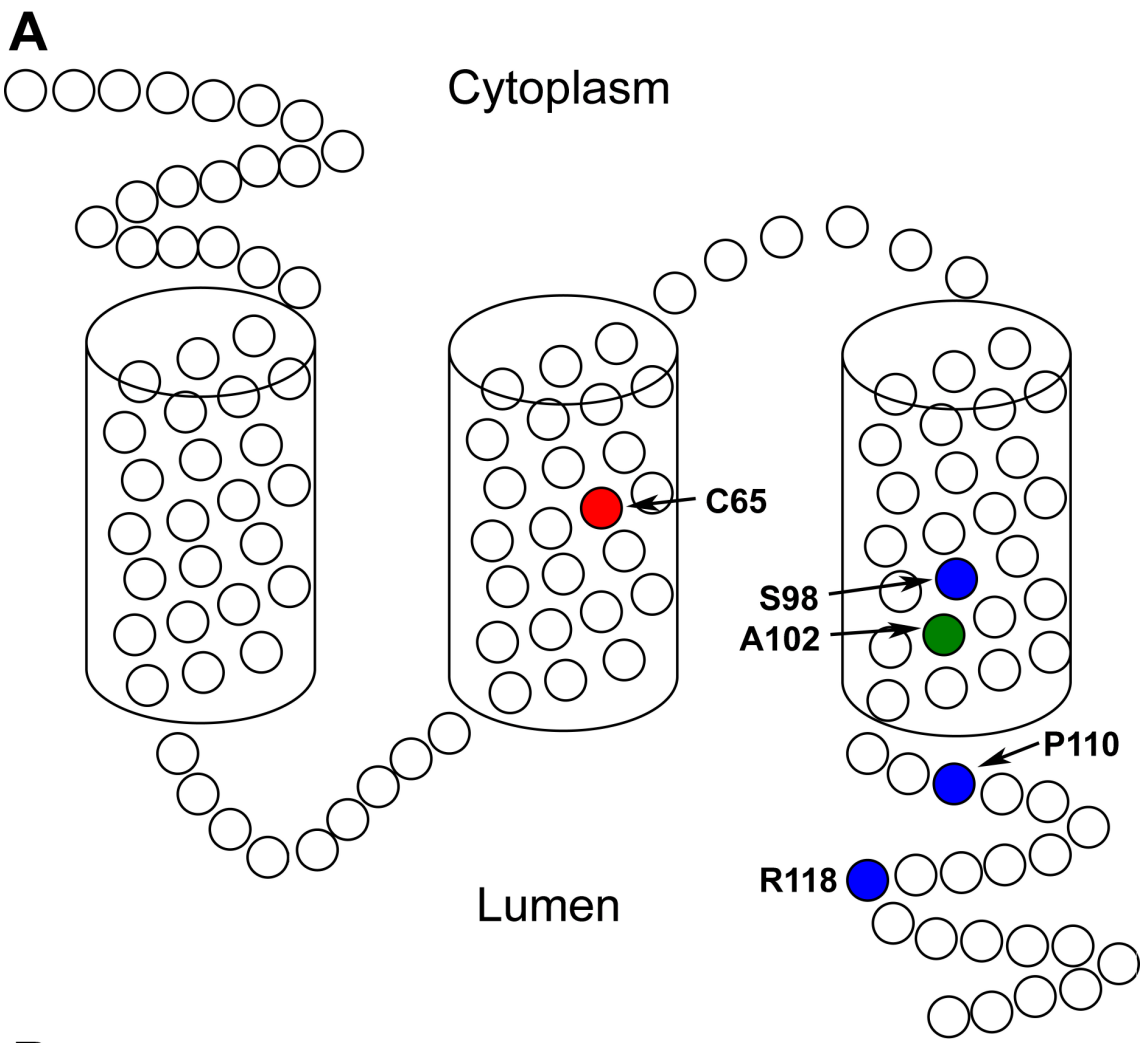


Figure 4 - The *etr1-11* mutation lies within the amino-terminal domain of *ETR1*. A) Diagram of the ETR1 ethylene-binding domain. Residues 1-128 of ETR1 are represented. The three cylinders represent transmembrane helices. Highlighted are C65 (red), which is mutated in *etr1-1*, A102 (green), which is mutated in *etr1-2*, and S98, P110, and R118 (blue), which are missense loss-of-function alleles of *ETR1* (this

study, Wang et al., 2006). B) Alignment of ethylene receptors across the plant kingdom. All five *Arabidopsis* ethylene receptors are shown (AtETR1, AtERS1, AtETR2, AtERS2, AtEIN4), as well as ethylene receptors from tomato (LeNR), rice (OsERS1), maize (ZmERS1), and *Synechocystis* (SspERS1). Highlighted are the loss-of-function mutations analyzed in this study. Asterisk represents the residue mutated in *etr1-11* plants, which is mutated to a tryptophan residue.

of-function allele. The arginine which is replaced in *etr1-11* plants is conserved in all five *Arabidopsis* ethylene receptors and in ethylene receptors known in tomato, rice, and maize, but not in ethylene-binding domains present in cyanobacterial (Figure 4A). Arg118 is located within the amino-terminal portion of ETR1, but is not located within any of the three transmembrane helices that contain the residues necessary for ethylene-binding (Figure 4B).

To examine the extent to which the *etr1-11* mutation is a loss-of-function, the *etr1-11* mutant (carrying the *etr1-2* allele) was crossed with the *ers1-3* mutant. The *ers1-3* mutation is a null allele of the *ERS1* ethylene receptor gene, and the *etr1-7 ers1-3* double mutant displays severe constitutive ethylene responses, including severely diminished rosette size, sterility, and a constitutive seedling triple response (Hall and Bleecker, 2003; Qu et al., 2007). The resulting *etr1-11 ers1-3* double mutant displayed a partial constitutive ethylene response when assayed for the triple response (Figure 5A). It did not, however, display the reduced rosette size and sterility seen in other *etr1 ers1* mutants (Figure 5B). While the *etr1-11 ers1-3* double mutant does not display the severe phenotypes of other *etr1 ers1* mutants, the partial constitutive triple response exhibited by the *etr1-11 ers1-3* mutant is a stronger ethylene response than is exhibited by either of the single *etr1* or *ers1* loss-of-function mutants.

To further test whether the *etr1-11* mutant represents a null allele of *ETR1*, the *etr1-11* mutation was introduced into an *ETR1* transgene. This transgene is an *ETR1* cDNA fused to the native *ETR1* promoter region and first intron of *ETR1* (Dong et al., 2008). The *etr1-11* mutation was introduced into the *ETR1* transgene by site-

Figure 5 - The *etr1-11 ers1-3* double mutant has partial constitutive ethylene responses. A) Etiolated seedlings were grown on MS plates for four days and representative seedlings photographed. Scale bar represents 1 mm. B) Plants were grown for three weeks on soil, then photographed. Scale bar represents 5 mm.

directed *in vitro* mutagenesis. The resulting *etr1-11* transgene was then introduced into the triple ethylene receptor null *etr1-6 etr2-3 ein4-4* background via *Agrobacterium*-mediated transformation. The *etr1 etr2 ein4* triple receptor null displays constitutive ethylene responses and is rescued by introducing the *ETR1* gene (Hua and Meyerowitz, 1998; Qu and Schaller, 2004). When the resulting transformants were assayed for the triple response in the absence of ethylene, it was found that plants transformed with the wild-type *ETR1* transgene rescued the constitutive response phenotype of *etr1 etr2 ein4* plants, whereas the R118W *ETR1* transgene failed to rescue the phenotype of the *etr1 etr2 ein4* genetic background (Fig. 6). This indicated that the *etr1-11* mutation knocks-out *ETR1* function independent of the *etr1-2* mutation.

etr1 loss-of-function mutations cannot suppress _etr1-1_, but suppress _etr1-2_

As mentioned previously, loss-of-function mutations in *RTE1*, a positive regulator of *ETR1* function, can suppress the *etr1-2* mutation, but not the strong ethylene-insensitive *etr1-1* mutation. Therefore, to assess whether the *etr1-11* mutation is essential for *etr1-1* receptor signaling, the *etr1-11* (R118W) mutation was introduced into an *etr1-1* transgene. The *etr1-1* transgene was created by introducing the *etr1-1* mutation into the *ETR1* transgene used in the previous study through *in vitro* mutagenesis. It has previously been shown that the introduction of an *etr1-1* transgene will confer ethylene-insensitivity to *Arabidopsis* plants transformed with the transgene (Gamble et al., 2002). The R118W *etr1-1* transgene was stably transformed into wild-type and *etr1-7 Arabidopsis* plants, and T_3 plants were analyzed for ethylene response by utilizing the triple response assay. The R118W

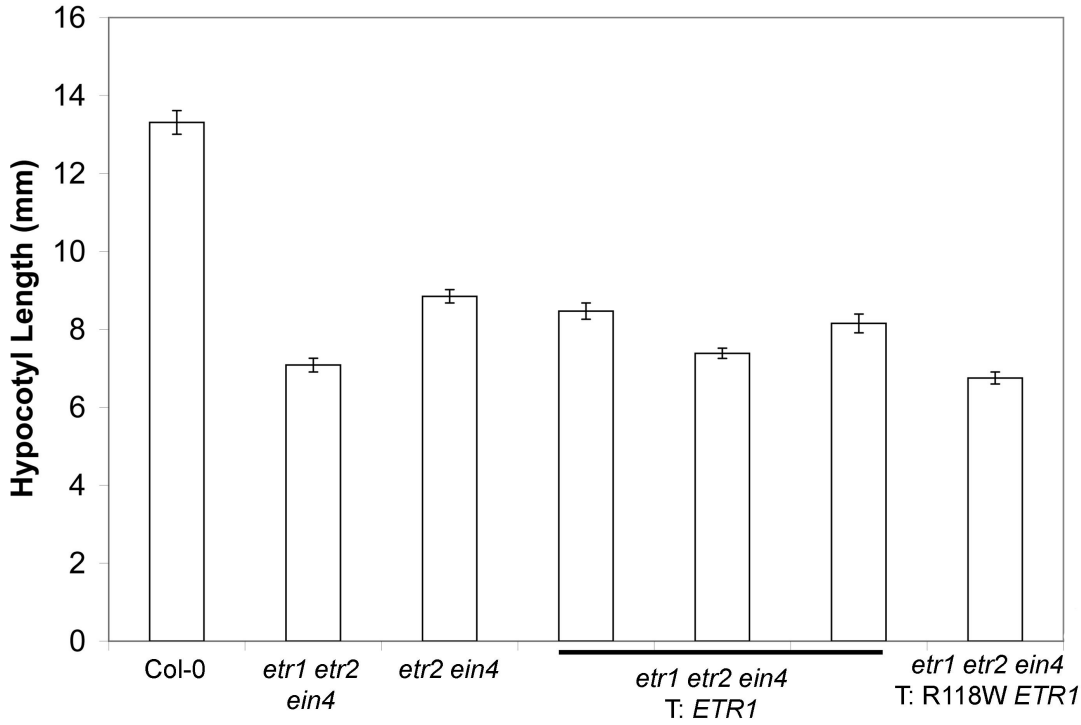


Figure 6 - The R118W mutation abrogates wild-type *ETR1* function. Etiolated seedlings were grown on MS plates for four days and representative seedlings photographed and measured. Data represents one experiment, where n = 20 for each data point. Error bars represent standard error of the mean.

etr1-1 transgene was able to confer such ethylene-insensitivity in three independent lines (Fig. 7). This indicates that the *etr1-11* mutation is unable to suppress the strong ethylene-insensitive *etr1-1* allele.

Previously, through a targeted mutagenesis approach, the S98A and P110A mutations were shown to be loss-of-function mutations in wild-type ETR1 receptors (Wang et al., 2006). To assay whether these mutations could suppress the ethylene-insensitive *etr1-1* and *etr1-2* mutations, the S98A and P110A mutations were introduced into *etr1-1* and *etr1-2* transgenes. When assayed by the triple response, the S98A and P110A mutations could not suppress the ethylene insensitivity of *etr1-1*, similar to the *etr1-11* mutation (Fig. 7). However, both the S98A and P110A mutations could suppress the ethylene-insensitivity of the *etr1-2* mutation (Fig. 7).

Discussion

In this study we have identified a new loss-of-function allele of the ethylene receptor gene *ETR1*. This new allele, *etr1-11*, is unique because it is the first missense loss-of-function mutation within any ethylene receptor gene isolated in a mutagenic screen. Most mutations in the ethylene receptors were isolated with simple mutagenic screens for ethylene insensitivity and are dominant, gain-of-function mutations. In fact, the first mutants obtained for each of the ethylene receptors were dominant, ethylene-insensitive mutations (Bleecker et al., 1988; Chang et al., 1993; Hua et al., 1995; Sakai et al., 1998; Hua et al., 1998). The paucity of loss-of-function mutants in the ethylene receptors is due, in part, to the redundancy of the receptors. The subtle nature of loss-of-function alleles of the ethylene receptors means that all previous loss-of-function alleles (and this one) have been

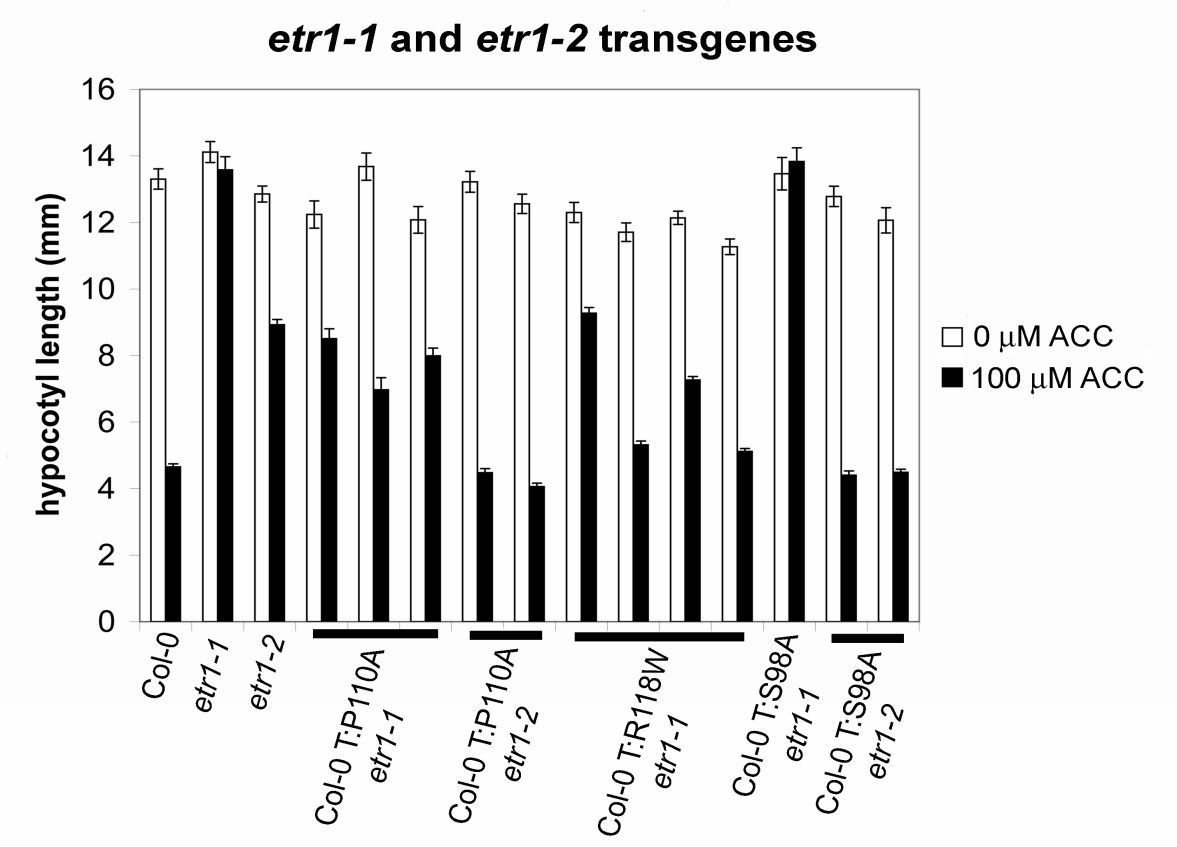


Figure 7 - The loss-of-function mutations R118W, S98A, and P110A suppress *etr1-2*, but not *etr1-1*. Etiolated seedlings were grown on MS plates with the indicated dose of ACC for four days. Seedlings were photographed and measured. Each data point represents an independent transformation line. Data is for one independent experiment where each data point is n = 20. Error bars represent standard error of the mean.

identified through screens for suppressors of ethylene-insensitive mutants or by designed *in vitro* mutagenesis. However, all other previous loss-of-function mutants isolated by mutant screening have been mutations that lead to a truncated protein. In fact, some of the null alleles in the ethylene receptors lead to reduced transcript levels, presumably through nonsense-mediated decay (Zhao et al., 2002; Cancel and Larsen, 2002). Additionally, in the study by Wang et al. in 2006 where residues within the ethylene-binding domain were targeted for *in vitro* mutagenesis, out of 41 mutants analyzed, only the S98A and P110A mutations gave a loss-of-function phenotype.

The apparent rarity of loss-of-function mutants within the ethylene receptors may be a clue to how the receptors function. It suggests that many more residues within the receptors are important for turning the receptors 'off' than for maintaining the receptors in the 'on' state. Interestingly, all three of these mutations are located in proximity to each other, either in the third transmembrane helix or in the cytoplasmic region immediately C-terminal to the transmembrane helices. This may indicate that this region is important for maintaining the receptor in an 'on' signaling state, although other mutations examined in this region (*etr1-2*, T101A, L105A, I108A, K116A among others) are either ethylene-insensitive or have no effect on ethylene signaling (Wang et al., 2006). The *etr1-11* mutation itself is a non-reciprocal one, as the arginine present in wild-type receptors is a positively charged amino acid, while the tryptophan present in the mutant is a large, hydrophobic residue. The impact of this change could be a large conformational change as the new hydrophobic residue changes position to obtain a lower energy state, destroying ETR1 function.

The experiments we have performed show that Arg118 is required for *etr1-2* function. The *etr1-11* mutation completely suppresses ethylene-insensitivity in the *etr1-2* background. The fact that the *etr1-11 ers1-3* double mutant displays constitutive ethylene responses for the triple response but does not exhibit the severe growth defects seen in the *etr1-7 ers1-3* mutant is interesting. When comparing the lesions in the *etr1-7* and *etr1-11*mutants, this difference makes sense, since the *etr1-7* mutation is a nonsense mutation, while *etr1-11* is a missense mutation. Why the *etr1-11* mutant has a strong hypersensitive response, comparable to the *etr1-7* mutant, while the *ers1-3* double mutants show a larger difference in phenotype is unknown. It has been proposed that *ETR1* has separable functions in plant growth and ethylene signaling (Cho and Yoo, 2007). It is possible that *etr1-11* reduces the ethylene signaling functions of ETR1, but not plant growth regulation functions.

Transgenic analysis showed that R118 is also required for wild-type ETR1 function as well as *etr1-2* function in one transformed line. It will be necessary to obtain and characterize more transformed lines to confirm the loss-of-function phenotype. Additionally, for all lines characterized, a Western blot will need to be performed to confirm expression of the transgenes. It had been shown in Wang et al. (2006), that S98 and P110 are required for wild-type ETR1 function. Consistent with R118, mutations in these two residues also disrupted *etr1-2*, suggesting that all three are needed for both wild-type and *etr1-2* signaling.

Perhaps most interesting is the fact that none of these three mutations could suppress *etr1-1* function, which suggests a novel signaling mechanism for *etr1-1* receptors. The fact that S98A, P110A, and R118W cause loss-of-function phenotypes

indicates that *ETR1* requires these residues for its function. The fact that *etr1-1*, which is a constitutively active mutant, does not require these residues indicates that *etr1-1* can signal in a manner different than *ETR1*. How *etr1-1* signals independently of normal ethylene receptor mechanisms remains unknown.

Materials and Methods

Plant Growth and Conditions - Unless otherwise stated, all *Arabidopsis* plants used in this study are of the Columbia (Col-0) ecotype. The *etr1-9* and *ers1-3* mutans are of the Wassilewskija (Ws) ecotype. For seedling growth and triple response assays, seeds were plated onto Murashige and Skoog (MS) (Sigma Aldrich) media containing 0.8% agar. Where indicated for ethylene response and ethylene inhibitor assays, plates also contained 100 μ*M* ACC, 100 μ*M* $AgNO_3$, or 10 μ*M* AVG (Sigma Aldrich). Plates were cold stratified at 4°C for three days, followed by growth at 20°C in the dark for four days. For ethylene dose response assays, plates were placed in airtight jars with indicated concentrations of ethylene gas (Airgas), and jars were kept in dark for four days. Seedlings were photographed and hypocotyl lengths were measured using ImageJ software (http://rsbweb.nih.gov/ij/). For soil-grown plants, seeds were either sown directly onto soil and cold stratified for 3 days at 4°C, or seedlings were transferred from plates. Plants were grown on MetroMix 360 Growing Medium (SunGro Horticulture) under cycles of 16 hours light and 8 hours dark at 22°C in light and 20°C in dark.

Mutagenesis and Mutant Screening – For mutagenesis, 200 mg of seeds of the *etr1-2* genotype were washed in a solution of 0.1% Tween 20 (Sigma Aldrich). Seeds were then incubated in a solution of 0.3% EMS (Sigma Aldrich) for 12 hours with

gentle agitation. Seeds were washed twice with water and suspended in 0.1% agarose. Seeds were sown onto soil and allowed to grow to maturity. Seeds were collected from these M_1 plants and plated onto 100 μ*M* ACC plates (described above) for screening. Suppressor mutants were isolated based on the exhibition of the triple response.

Senescence Assays – For cotyledon senescence assays, 17-day old plants were placed in transparent airtight chambers with either air or 100 ppm ethylene. After three days of treatment, plants were removed and representative cotyledons were photographed. For adult plant senescence, four-week old plants were placed in airtight chambers with either air or 100 ppm ethylene. After four days of treatment, plants were removed and photographed.

***In vitro* Mutagenesis and Plant Transformation -** *etr1-1*, *etr1-2*, S98A *etr1*, P110A *etr1*, and R118W *etr1* transgenes were generated using the QuikChange II XL Site-directed Mutagenesis kit (Stratagene). The *ETR1*-5xMyc construct in pMLBart (Dong et al., 2008) was used as template for the mutagenesis for *etr1-1* and *etr1-2*, and R118W *etr1* transgenes. For S98A *etr1-1*, P110A *etr1-1*, and R118W *etr1-1* transgenes, the *etr1-1* transgene construct was used as template for mutagenesis. For S98A *etr1-2* and P110A *etr1-2* transgenes, the *etr1-2* transgene construct was used as template for mutagenesis. Primers used: S98A *etr1-1*: 5' GTTAACCGCTGTTGTCG CGTGTGCTACTGCGTT 3' and 5' AACGCAGTAGCACACGCGACAACAGCGG TTAAC 3'. S98A *etr1-2*: 5' TGTTAACCGCTGTTGTCGCGTGTGCTACTACGTT G 3' and 5' CAACGTAGTAGCACACGCGACAACAGCGGTTAACA 3'. P110A *etr1-1*: 5' ACTGCGTTGATGCTTGTTCATATTATTGCTGATCTTTTGAGTG 3'

and 5' CACTCAAAAGATCAGCAATAATATGAACAAGCATCAACGCAGT 3'. P110A *etr1-2*: 5' GCTACTACGTTGATGCTTGTTCATATTATTGCTGATCTTTT GAGTGT 3' and 5' ACACTCAAAAGATCAGCAATAATATGAACAAGCATCAA CGTAGTAGC 3'. R118W *etr1* and R118W *etr1-1*: 5' ATTCCTGATCTTTTGAGT GTTAAGACTTGGGAGCTTTTCTTG 3' and 5' CAAGAAAAGCTCCCAAGTCTT AACACTCAAAAGATCAGGAAT 3'. All constructs were transformed into the *Agrobacterium tumefaciens* strain GV3101, which was then used to infect the indicated *Arabidopsis* genotypes. Transformants were selected for with Basta (0.033% Liberty herbicide, Bayer Cropscience).

Isolation and characterization of *rte3-1*, a suppressor mutant of *etr1-2*

Introduction

The ethylene signaling pathway has been studied in detail mostly through the use of genetic screens. Most of the genes involved in the transmission of the ethylene signal have been identified by mutagenic screens for either insensitive or constitutive ethylene responses. Through epistasis and molecular analysis of the genes involved, a mostly linear pathway has emerged from receptor to induction of gene expression. However, the simple search for ethylene insensitive or constitutive response mutants may have become saturated, since these screens yield multiple alleles of the same genes.

With this in mind, our lab undertook a unique genetic screen for discovering novel regulators of ethylene signaling. This screen involved searching for suppressors of the dominant ethylene-insensitive mutant *etr1-2*. The weak ethylene insensitivity of the *etr1-2* allele makes it a good target for mutagenesis, since it allows screening for both suppressor and enhancer mutations. However, since one outcome of an enhancer screen would most likely be ethylene-insensitive mutants already isolated by previous screens, we have done screens solely for suppressors of *etr1-2* insensitivity. The suppressor screen is ideal for finding mutants not found by previous screens, due to the redundancy of the ethylene receptors. A mutant that causes loss of ethylene receptor function would likely be masked by the other

receptors, so by assaying for the ability to suppress the insensitivity of a single receptor may uncover mutants that have not been found before.

This search has borne fruit in the past, as the *rte1* mutant was isolated in this manner (Resnick et al., 2006). It would be unlikely to isolate *rte1* in a traditional insensitivity or constitutive response screen, as *rte1* loss-of-function mutants exhibit only slight ethylene hypersensitivity in the absence of the *etr1-2* allele (Resnick et al., 2006). Furthermore, since *rte1* is a regulator specific to *ETR1*, the use of a suppressor screen yielded an interaction that is unique to the ETR1 ethylene receptor. Therefore, the use of a suppressor screen with *etr1-2* is a proven option for discovering heretofore uncharacterized members of the ethylene signaling pathway.

To repeat the suppressor screen, Mandy Kendrick, a former graduate student in the Chang lab, mutagenized a population of *etr1-2* seeds, and Dr. Chun-Hai Dong, a current post-doctoral researcher in the lab, performed another suppressor screen on the mutagenized seed population. Among several mutants isolated from this screen, one was named *reversion to ethylene-sensitivity3* (*rte3*), and was crossed to *etr1-2* to test for dominance or recessiveness. The *rte3* mutant was also crossed to wild type to test if the suppressor mutation was intragenic or extragenic. The results of these crosses showed that *rte3* was a recessive, extragenic suppressor mutant of *etr1-2* (data not shown). This raised the question of where *rte3* acted within the ethylene signaling pathway to suppress *etr1-2* function and the mechanism by which it suppresses *etr1-2* function. To answer these questions, I began to study the *rte3* mutant in greater detail.

Results

RTE3 is unlikely to be a member of the ethylene signaling pathway.

To gain an understanding of the role of *RTE3* in ethylene signaling, the ethylene responses of the *rte3-1* mutant and the *rte3-1 etr1*-2 mutant were characterized. When treated with the ethylene biosynthetic precursor ACC, *rte3-1 etr1-2* etiolated seedlings exhibited an exaggerated apical hook and a shortened and thickened hypocotyl, characteristic of the triple response (Fig 8A). However, the hypocotyl of this mutant was not as short as the wild type when treated with ACC and did not have the shortened root that wild-type seedlings exhibit when treated with ethylene. In addition, the *rte3-1* single mutant, with a wild-type copy of *ETR1*, displayed an extremely short hypocotyl in response to ACC, reminsicient of the ethylene hypersensitive mutant *etr1-7*. However, this was also accompanied by an overall shorter hypocotyl in the *rte3-1* mutant, so it was impossible to conclude that the *rte3-1* mutant exhibits ethylene hypersensitivity from this data alone.

The intermediate phenotype of *rte3-1 etr1*-2 suggested a partial suppression of the *etr1-2* allele, so to characterize the response further, the single and double mutants were subjected to an ethylene dose response assay (Fig. 8B). When treated with doses of ethylene gas ranging from 0.01 to 100 ppm, the *rte3-1 etr1-2* mutant displayed a response curve intermediate to the wild type and the *etr1-2* mutant. The *rte3-1* single mutant displayed a dose response that appears to be hypersensitive to ethylene, but the *rte3-1* mutant still displays a short hypocotyl when not treated with ethylene. One possible reason that these phenotypes could occur is that the *rte3-1* mutation may cause a defect in hypocotyl growth.

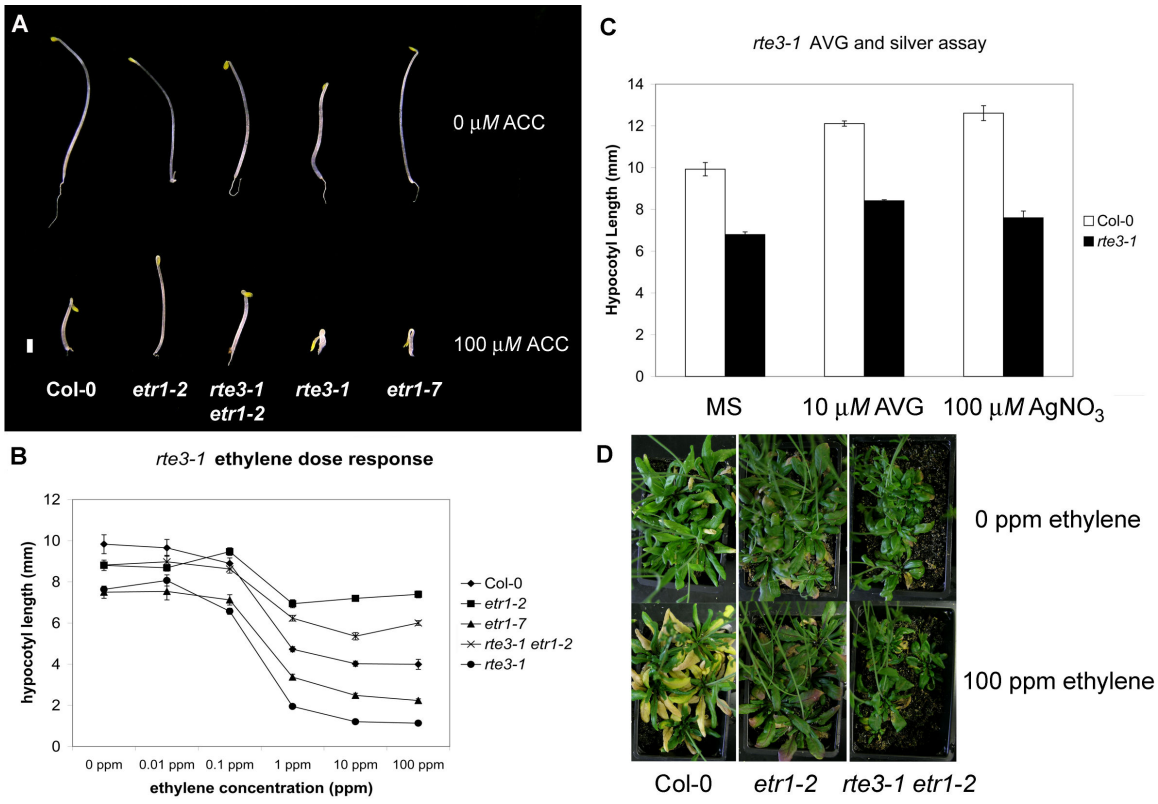


Figure 8 – Ethylene responses of *rte3-1*, *rte3-1 etr1-2*, and *etr1-7*. A) Etiolated seedlings were grown for four days on MS plates with the indicated dose of ACC. Representative seedlings were sampled and photographed. Scale bar represents 1 mm. B) Etiolated seedlings were grown for four days in air-tight jars with the indicated dose of ethylene gas. Seedling hypocotyls were photographed and measured. Error bars represent standard error of the mean. Data represents one experiment where n = 12 for each data point. C) Etiolated seedlings were grown for four days on MS plates with the indicated treatment. Seedlings were photographed and measured. Error bars represent standard error of the mean. Data represents one experiment where n = 12 for each data point. D) Four-week old plants were placed in air-tight chambers and treated for four days with the indicated dose of ethylene or air. Plants were removed and photographed.

To test whether the *rte3-1* defect is in ethylene response or in etiolated seedling growth, the *rte3-1* mutant was grown on AVG, an ethylene biosynthesis inhibitor, and on silver nitrate, an ethylene response inhibitor. If the short hypocotyls of *rte3-1* are due to a hypersensitive response to endogenously produced ethylene, then treatment with AVG or silver should block this response. However, a general growth defect will not be rescued by treatment with these chemicals. When treated with AVG or silver nitrate, the *rte3-1* mutant was not rescued to the wild-type phenotype, suggesting that *rte3-1* does not exhibit an ethylene phenotype (Fig. 8C). To confirm this hypothesis, the *rte3-1 etr1-2* mutant was tested for another ethylene phenotype, adult leaf senescence. When *rte3-1 etr1-2* adult plants were treated with 100 ppm ethylene for four days, their leaves did not senesce, showing that *rte3-1* does not suppress *etr1-2* ethylene insensitivity when assayed by leaf senescence (Fig. 8D). These results suggest that *rte3-1* does not have an ethylene response phenotype, but instead has a seedling growth phenotype.

The *rte3-1* mutation was crossed into several other ethylene-insensitive receptor mutations to discover if the defect seen in *rte3-1 etr1-2* is specific to *ETR1*. The *rte3-1* mutation was crossed to the strong ethylene-insensitive *ers1-1* and *etr2-1* alleles, as well as the weakly ethylene-insensitive *ers1-10* allele (Hua et al., 1995; Sakai et al., 1998; Alonso et al., 2003). The *rte3-1* mutation was unable to suppress the *ers1-1* and *etr2-1* mutations as assayed by the triple response (Fig. 9). However, the *rte3-1 ers1-10* double mutant was significantly shorter than the *ers1-10* mutant alone. One possible explanation for this data is that the *rte3-1* mutation causes a hypocotyl shortening that is overridden in strong ethylene insensitive mutants like

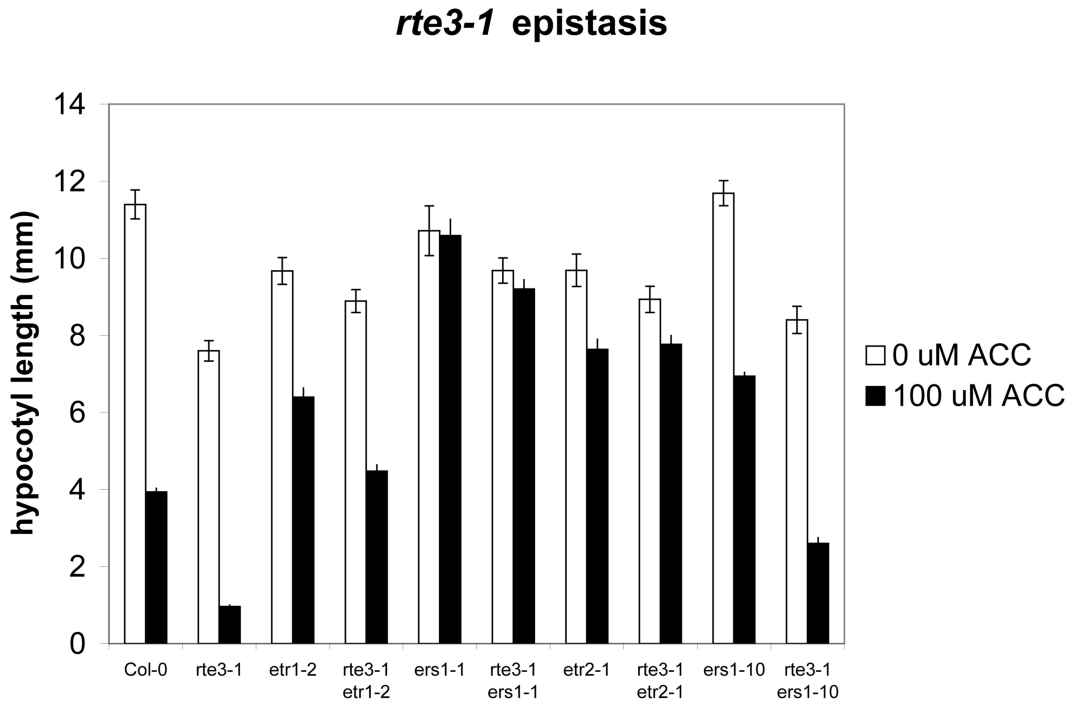


Figure 9 – *rte3* does not suppress other ethylene-insensitive receptor alleles. *rte3-1* was crossed into the indicated genotypes and homozygous double mutant lines were obtained. Etiolated seedlings were grown on MS plates with the indicated treatment for four days, then photographed and hypocotyls measured. Error bars represent standard error of the mean. Data represents one experiment where n = 20 for each data point.

ers1-1 and *etr2-1*, while weakly ethylene-insensitive mutants like *etr1-2* and *ers1-10* are unable to override the defect of the *rte3-1* mutation due to their weaker signal. While it may suggest a role for *RTE3* in ethylene signaling, it is more likely that the weak signaling strength of *etr1-2* and *ers1-10* are not able to override the lack of a growth signal in *rte3* mutant hypocotyls, while the *ers1-1* and *etr2-1* mutants are able to override the *rte3* defect due to the stronger signal present in these mutants.

A short hypocotyl may be due to a reduction in cell size or cell number. To assay whether the *rte3-1* mutant had reduced cell size, hypocotyls of dark-grown seedlings were photographed using a compound microscope and cell size was measured using an imaging program. When quantified, cells in *rte3-1* etiolated hypocotyls were the same size or slightly larger than wild-type hypocotyls (Student's t-test: $0.05<p<0.10$) (Fig. 10). This suggests that the *rte3-1* mutant has a defect in cell number, rather than cell size. This could be an indicator that *rte3-1* plants have a slower cell cycle, leading to fewer cell divisions, and hence, fewer cells.

Molecular cloning of the *RTE3* gene

To uncover the *RTE3* gene product and fully understand the defect in the *rte3* mutant, map-based cloning was performed to isolate the *RTE3* gene. For the mapping cross, the *rte3-1 etr1-2* mutant, which was generated in the Columbia (Col-0) ecotype, was crossed to an *etr1* mutant in the Landsberg-erecta (Ler) background. This cross eliminated the problem of having wild-type seedlings segregating in the F_2 mapping population, as they would have the same ethylene responses as the suppressed *rte3-1 etr1-2* double mutant seedlings. The F_2 progeny from this cross

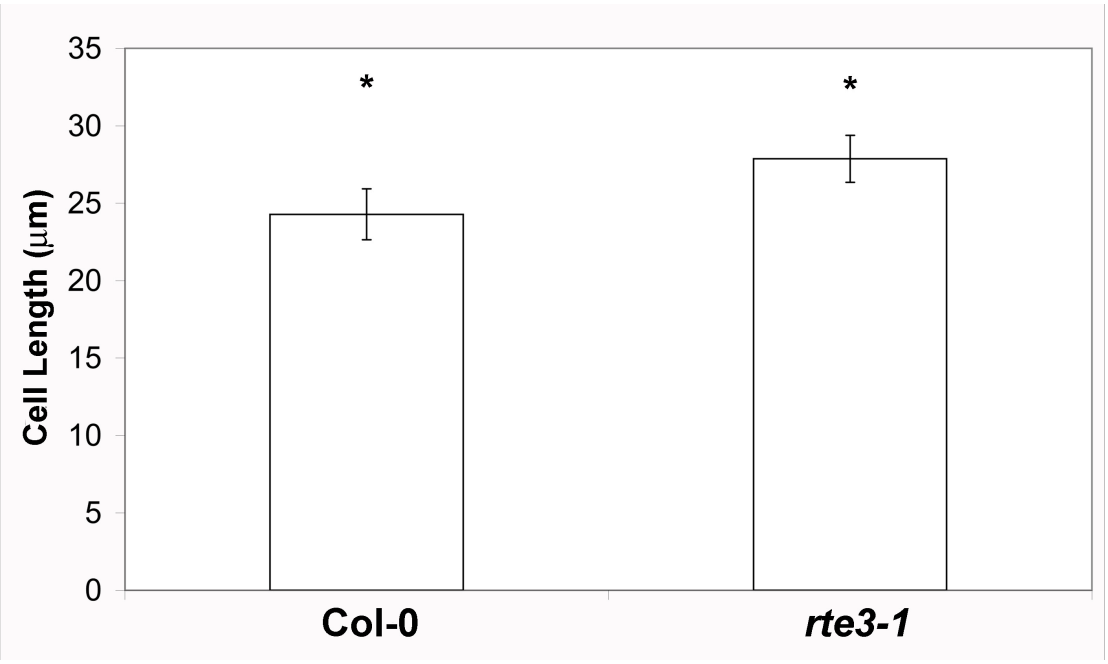


Figure 10 - Etiolated *rte3-1* hypocotyl cells are the same size as wild-type hypocotyl cells. Seedlings were grown in the dark for four days on MS media, then hypocotyls were imaged with a Nikon Labphot-2 microscope equipped with a Zeiss IcC3 Axiocam. Cell lengths were measured using the imaging software ImageJ. For one experiment where n>30 cells for each genotype, stars indicate that cell lengths were not significantly different between wild type and *rte3-1* (Student's t-test, $0.05< p<0.10$).

were screened for the triple response phenotype on 100 μ*M* ACC, and responders were isolated to form a mapping population.

The mapping population was genotyped using several SSLP and SNP markers throughout the *Arabidopis* genome (generated from the Monsanto Ler polymorphism collection, http://www.arabidopsis.org/browse/Cereon/index.jsp). The *rte3-1* mutation was found to be linked to the upper arm of chromosome 3. After several rounds of isolating more members of the mapping population, designing genotyping markers, and testing the mapping population for these markers, the *RTE3* locus was found to lie within a 50 kb region carried by bacteria artificial chromosomes (BACs) F28L1 and F24P17 (Fig 11). Subsequent sequencing of the genes residing within this 50 kb region revealed a guanine to adenine transition mutation at nucleotide 6696 of At3g06290. This mutation leads to a stop codon within the predicted protein sequence at amino acid 1187. Subsequent sequencing of a suppressor mutant from the same screen that failed to complement *rte3-1* revealed a second allele, *rte3-2*, with another guanine to adenine transition within At3g06290, at nucleotide 7098. The *rte3-2* mutation is also a nonsense mutation, which changes tryptophan 1321 to a stop codon.

The *RTE3* gene is previously uncharacterized, and the genomic sequence is approximately 8.1 kb in length from start codon to stop codon. Subsequent cloning of the *RTE3* coding sequence by RT-PCR revealed that *RTE3* contains 19 exons and 18 introns, although no UTR sequences were cloned, so the UTR sequences are unconfirmed. Especially interesting to note is that the obtained coding sequence from RT-PCR differs from the annotated prediction (no supporting cDNA) in TAIR. The

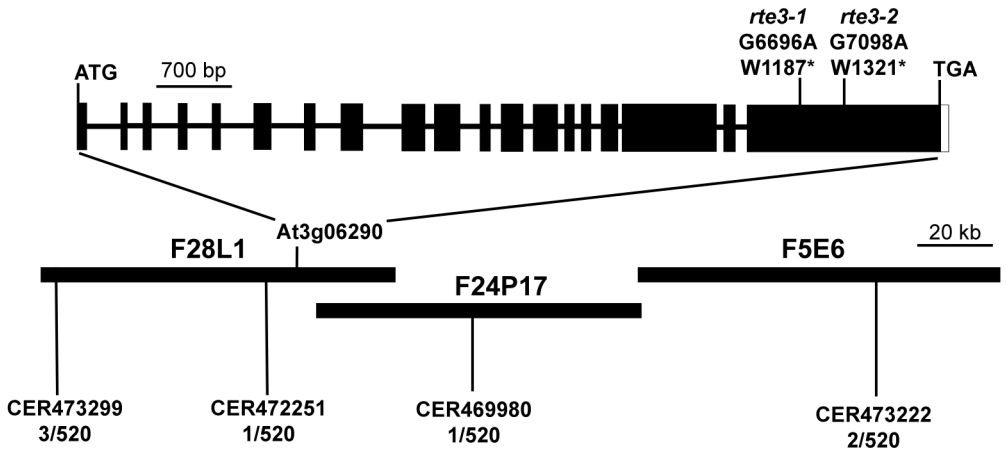


Figure 11 – The cloning of the *RTE3* gene. Based on map-based cloning techniques, the *rte3-1* mutation was found to lie within a 51 kb region on BACs F28L1 and F24P17. Sequencing revealed a nonsense mutation at nucleotide 6696 of At3g06290, a previously uncharacterized gene in *Arabidopsis*. The *RTE3* gene is approximately 8100 base pairs in length, and cloning of the *RTE3* coding sequence showed that the sequence contains 19 exons and 18 introns. Subsequent sequencing of another suppressor mutant that failed to complement *rte3-1* revealed another nonsense mutation, at nucleotide 7098. Both mutations change tryptophan residues to stop codons. In the bottom portion of the figure, black bars represent BAC clones, while lines and code numbers represent specific genotyping markers. Numbers represent number of recombinant chromsomes found out of the number of chromosomes tested. In the upper portion of the figure, black boxes represent exons, while connecting lines represent introns. White box represents the 3' UTR sequence of the transcript. Since only the coding region was amplifed by RT-PCR, the UTR sequences are those that are annotated in the *Arabidopsis* Information Resource (TAIR), http://www.arabidopsis.org.

annotated sequence contains 54 nucleotides of sequence (nucleotides 385 - 438 of the genomic sequence) at the 5' end of the second exon that are spliced out with the first intron in the cloned coding sequence. This leads to a segment of 18 amino acids that are predicted on the TAIR website, but would not be present in the cloned coding sequence. Interestingly, when cloning the sequence of *RTE3*, there were two alternative splice forms found. The additional splice form had an extra 215 nucleotides at the 5' end of the sixth exon (nucleotides 1428 - 1642 of the genomic sequence) that is spliced out in the first splice form. This second splice form leads to a frameshift in the *RTE3* cDNA, and a stop codon is soon encountered, producing a severely truncated protein. Therefore, the first splice form was used for all subsequent applications.

The predicted RTE3 protein is 1680 amino acids in length. According to CD search, the RTE3 protein has one conserved domain, which is a SAC3/GANP domain that has no known molecular function, located at residues 426-909 (Marchler-Bauer et al., 2002). The RTE3 protein is predicted to be a soluble protein based on the transmembrane predicting algorithm at ARAMEMNON (www.aramemnon.botanik.uni-koeln.de), and has no predicted sub-cellular localization. Using the predicted RTE3 protein sequence in BLAST analysis, there are proteins that contain an annotated SAC3/GANP domain in all eukaryotes. However, only plants contain proteins that show homology over the length of the entire RTE3 protein.

To confirm that the mutation mentioned above is responsible for the *rte3* phenotype, the genomic *RTE3* sequence was used to rescue the mutant phenotype.

The genomic sequence including the native *RTE3* promoter sequence 1.4 kb upstream of the start of the *RTE3* coding sequence was cloned into an expression plasmid containing a single myc epitope tag to be fused to the C-terminus of the RTE3 protein (Earley et al., 2006). This construct was transformed into *rte3-1 etr1-2* plants via *Agrobacterium*-mediated transformation. Homozygous transformed lines were isolated and T_3 lines were assayed for the triple response phenotype (Fig. 12). The genomic *RTE3* gene was able to rescue the *rte3-1 etr1-2* to the *etr1-2* ethylene-insensitive phenotype, demonstrating that the identified mutation is indeed responsible for the *rte3* phenotype.

Since the *rte3-1* and *rte3-2* mutations occur at the 3' end of the gene, it is possible that the *rte3* mutants have a semi-functional RTE3 protein product. To determine if *RTE3* expression is knocked-down or knocked-out in the mutant, RT-PCR analysis of the *RTE3* gene was performed. Although expression of *RTE3* appears to be reduced in the *rte3-1* mutant, it is not abolished completely, indicating that the *rte3-1* mutant is not a null (Fig. 13). One T-DNA line (*rte3-3*) was obtained from the *Arabidopsis* Biological Resource Center (ABRC) (WiscDsLox489-492C8) and tested for its ability to suppress *etr1-2*, but it did not show the *rte3* phenotype (Fig. 14). This is probably because the insertion is present within an intron of *RTE3* and may be spliced out of the transcript. Other T-DNA lines were ordered, but I was unable to isolate homozygous knock-outs lines due to genotyping difficulties.

To test whether the overexpressed *RTE3* gene would have the opposite phenotype as the *rte3* loss-of-function mutant, the *RTE3* coding sequence was cloned into a 35S expression vector and transformed into wild-type plants. Two independent

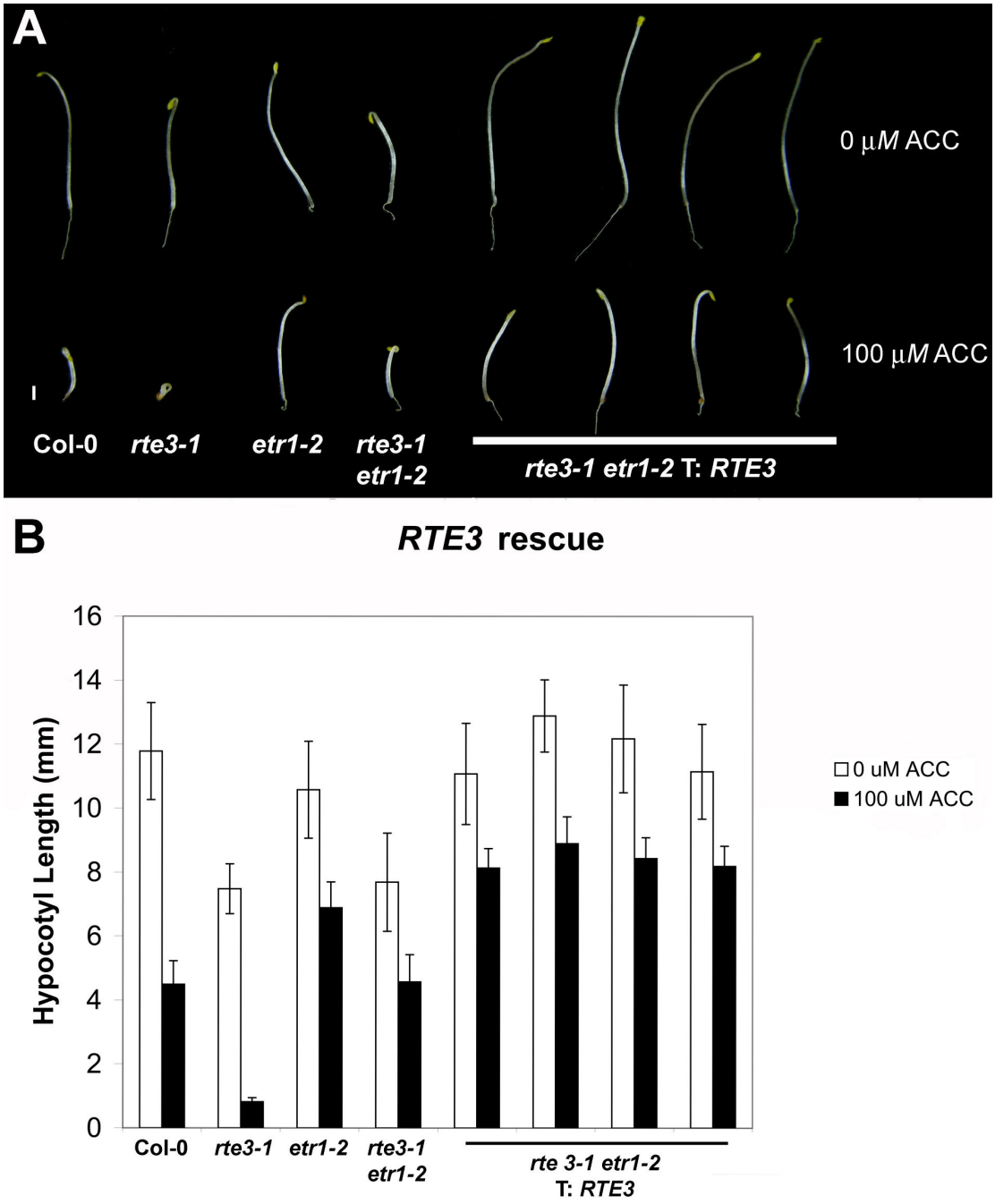


Figure 12 – A genomic *RTE3* construct rescues the *rte3* phenotype. The genomic sequence of *RTE3* including a 1.4 kb *RTE3* promoter fragment was cloned into pEarleyGate303 and transformed into *rte3-1 etr1-2* via *Agrobacterium* infiltration. A) Etiolated seedlings were grown on MS plates with the indicated dose of ACC for

four days, then representative seedlings photographed. Each seedling of *rte3-1 etr1-2* T: *RTE3* represents an independent transformation line. Scale bar represents 1 mm. B) Etiolated seedlings were grown on MS plates with the indicated dose of ACC for four days. Seedlings were photographed and measured. Error bars represent standard deviation. Data represents one experiment where n = 20 for each data point.

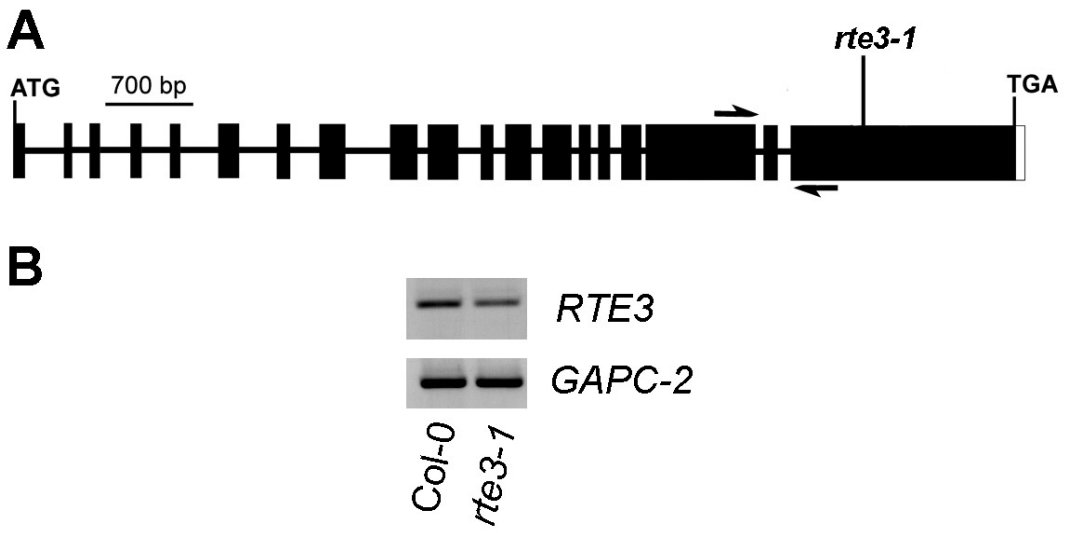


Figure 13 - RT-PCR of *RTE3* in the *rte3-1* mutant. A) Diagram of *RTE3* with location of RT-PCR primers indicated. B) Expression of the *RTE3* gene in wild-type and the *rte3-1* mutant. Total RNA was isolated from four-day old light-grown seedlings, and cDNA made using oligo(dT) primers. cDNA was used in PCR amplification of *RTE3* and *GAPC-2* (loading control) genes.

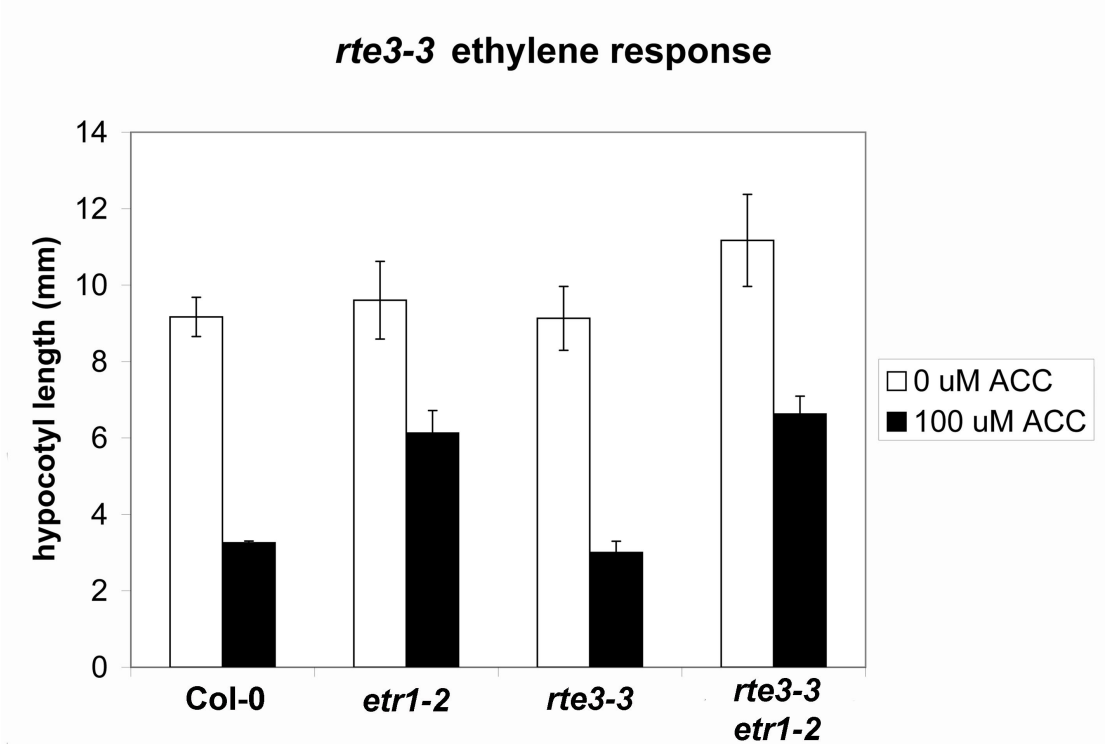


Figure 14 - Ethylene response of *rte3-3* and *rte3-3 etr1-2*. The *rte3-3* T-DNA insertion line (WiscDsLox489-492C8) was crossed to *etr1-2,* and homozygous plants were obtained. Etiolated seedlings of the above genotypes were grown on MS plates with the indicated ACC treatment for four days. Seedlings were photographed and measured. Error bars represent standard deviation. Data represents one experiment where n = 12 for each data point.

insertion lines displayed no phenotype in relation to seedling growth (results not shown).

The RTE3 protein is localized to the nucleus

The cellular role of *RTE3* in the regulation of seedling growth was unclear, so to discover what role the RTE3 protein may play in the cell, the sub-cellular localization of the RTE3 protein was determined. To accomplish this, the genomic *RTE3* construct with its native promoter was inserted into the pMDC107 expression vector (Curtis and Grossniklaus, 2003). This construct contains a GFP-6x-His tag placed at the C-terminus of RTE3. The RTE3-GFP expression vector was transformed into both *rte3-1 etr1-2* and wild-type plants. When assayed by the triple response phenotype, the RTE3-GFP fusion protein is able to rescue the *rte3-1 etr1-2* phenotype (Figure 15).

RTE3-GFP was localized using *Arabidopsis* roots in the wild-type background. Plants were grown either in 24-hour light or in the dark for four days, then their roots were imaged using confocal microscopy. When co-localized with the DNA stain 4',6-diamidino-2-phenylindole (DAPI), RTE3-GFP roots grown in the light localized to the nucleus (Figure 16). However, roots grown in the dark had almost no expression of RTE3-GFP, indicating that RTE3 protein may be regulated by light conditions (Figure 17). Control plants containing no transgene had no GFP expression (Figure 18). The presence of RTE3 in the nucleus may indicate that the protein may have a role in gene expression regulation or nuclear transport. It is especially interesting that RTE3 protein levels are regulated by light conditions, but opposite of what would be expected. The presence of a phenotype in the dark would

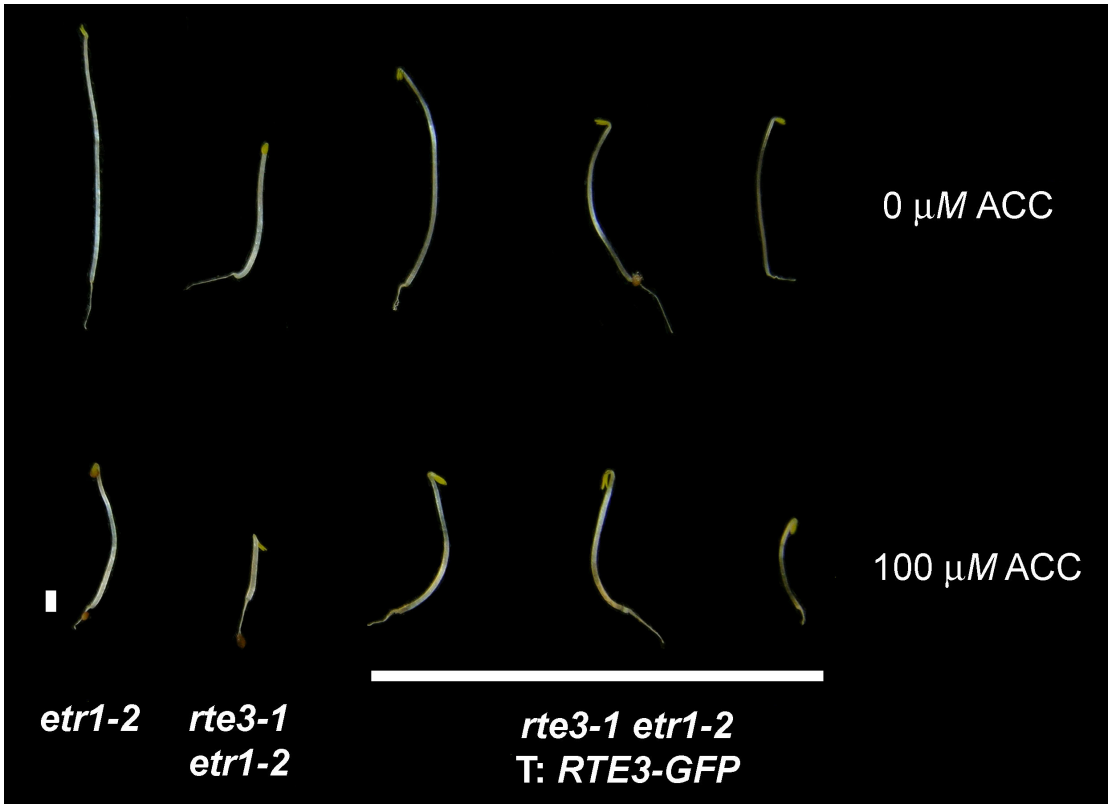


Figure 15 - The RTE3-GFP fusion protein is functional. A genomic *RTE3* construct with a 1.4 kb native promoter fragment was inserted into the GFP-6x-His tag expression vector pMDC107. This construct was transformed into *rte3-1 etr1-2* plants by *Agrobacterium* infiltration. Segregating T_2 lines were grown on MS plates with the indicated dose of ACC for four days in the dark. Three independent lines showed segregation of the rescued phenotype and representative individuals were photographed. Scale bar represents 1 mm.

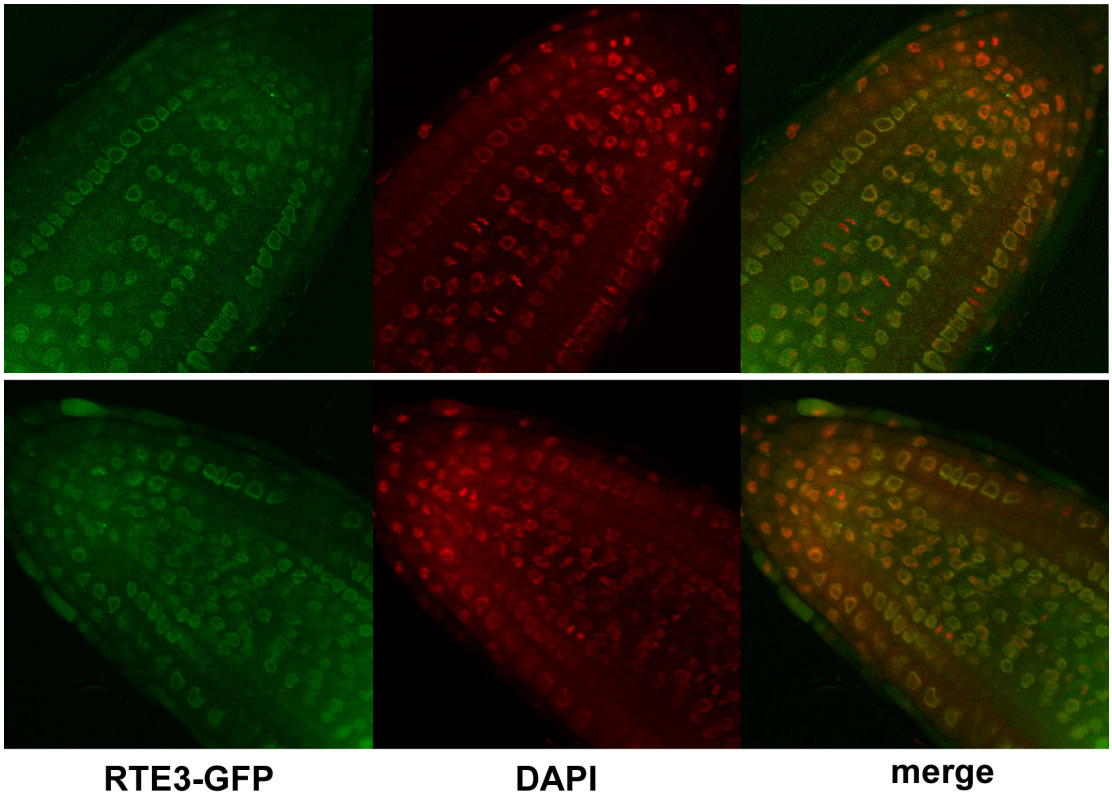


Figure 16 - RTE3-GFP is localized to the nucleus. Col-0 plants transformed with the genomic RTE3-GFP construct were grown under 24-hour light for four days. Roots were stained with 5 μg/mL DAPI for 20 minutes, then were imaged using an SP5 X confocal microscope.

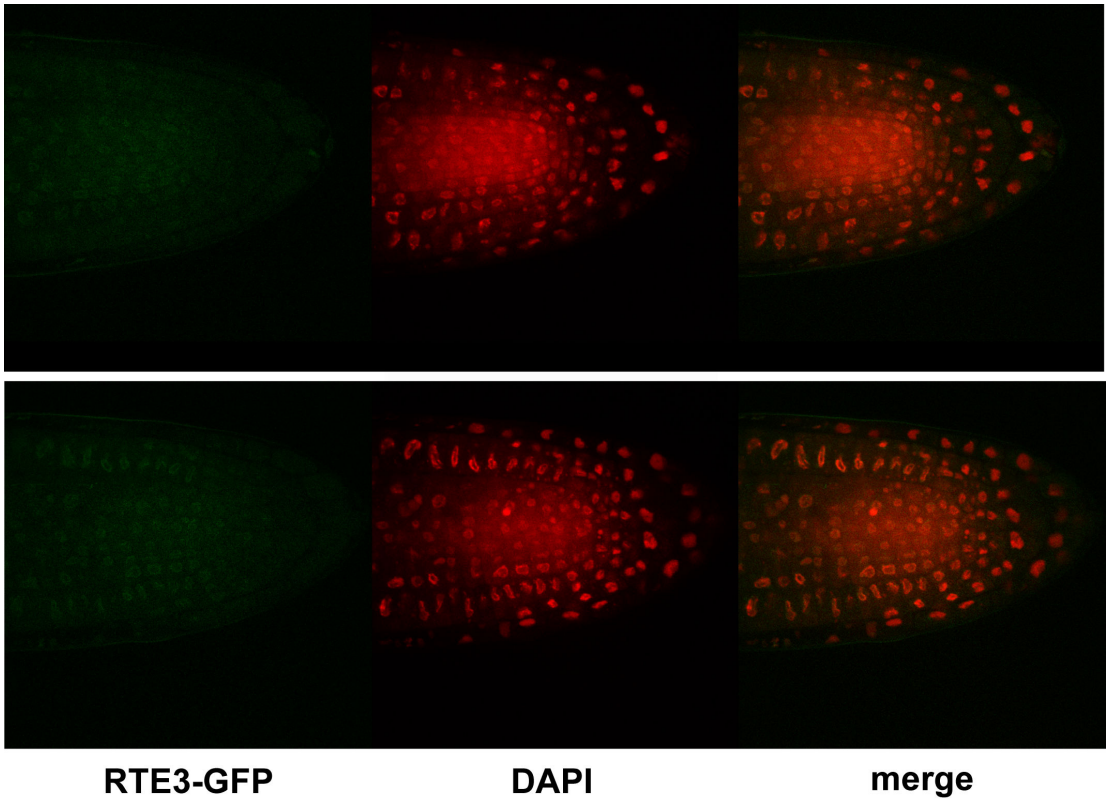


Figure 17 - RTE3-GFP expression is down-regulated in the dark. Col-0 plants transformed with the genomic RTE3-GFP construct were grown in the dark for four days. Roots were stained with 5 μg/mL DAPI for 20 minutes, then were imaged using an SP5 X confocal microscope.

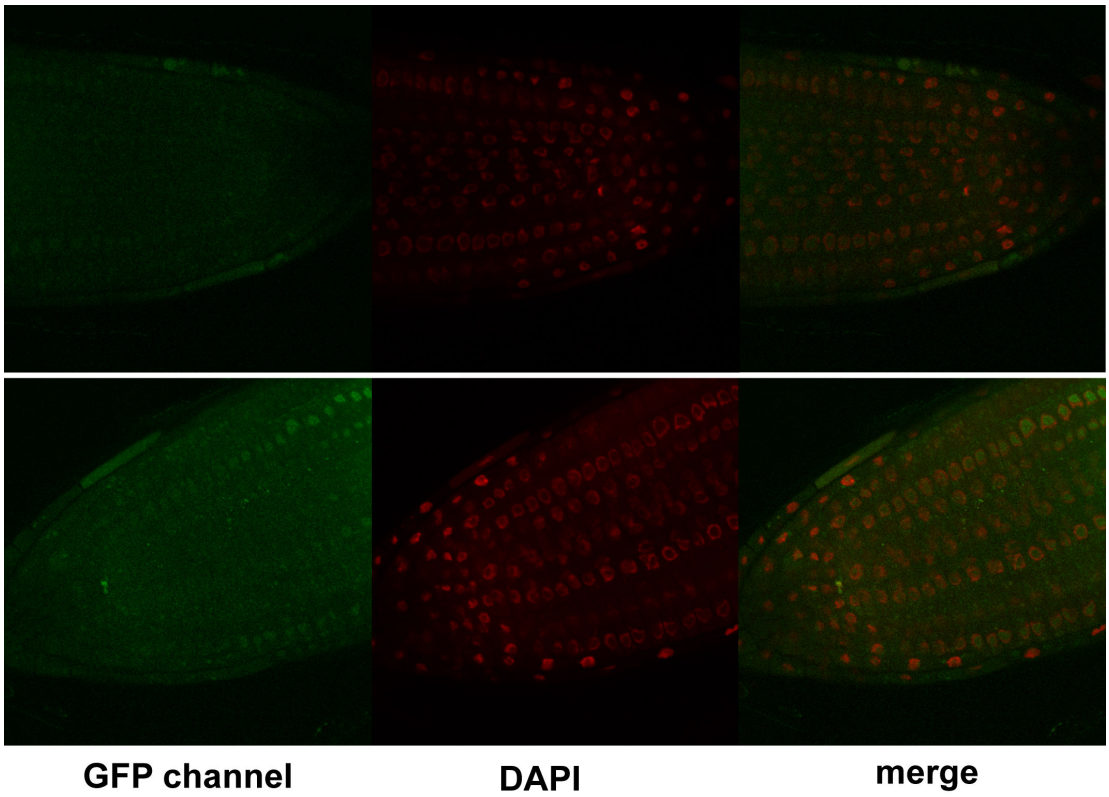


Figure 18 - Confocal microscopy of control plants. Untransformed Col-0 plants were grown under 24-hour light for four days. Roots were stained with 5 μg/mL DAPI for 20 minutes, then were imaged using an SP5 X confocal microscope.

suggest that RTE3 is expressed under this condition. One possible explanation would be that RTE3 expression could change depending on tissue type.

Discussion

The original intent of the screen for suppressors of *etr1-2* was to find new members of the ethylene signaling pathway. When initially isolated, based on its triple response phenotype, the *rte3-1 etr1-2* mutant appeared to possess a wild type response to ethylene treatments. However, closer examination of the triple response revealed only a partial suppression, especially in the roots of the examined seedlings. In addition, the single *rte3-1* mutant displayed a shorter hypocotyl, even in the absence of ethylene. These two phenotypes led to a greater examination of the *rte3* phenotype. When treated with the ethylene biosynthesis inhibitor AVG or the ethylene response inhibitor silver, *rte3* etiolated seedlings failed to rescue to the length exhibited by the wild type. This result showed that the hypocotyl length defect was most likely a result of a growth defect rather than a hypersensitive response to endogenously produced ethylene. Additionally, when assayed by a second ethylene phenotype, leaf senescence, *rte3-1* failed to suppress the insensitivity of *etr1-2*, demonstrating that *rte3* most likely does not display an ethylene phenotype.

The most likely explanation for the phenotypes shown by the *rte3-1*and *rte3-1 etr1-2* mutants is that *rte3-1* has a seedling growth defect. This defect is most likely confined to the hypocotyls of etiolated seedlings, since adult plants show no obvious morphological defects. Cell size is not affected in *rte3-1* hypocotyls, so the likely explanation for the shorter hypocotyls is a reduction in the number of cells. This could be explained by the slowing of the cell cycle machinery to produce fewer cell

divisions. In fact, cell size may be slightly larger in *rte3-1* hypocotyls, as a t-test indicated that the two classes are close to being significantly different. A reduced rate of cell division would lead to larger cells, supporting the hypothesis that *rte3-1* could have a reduced rate of cell division.

The hypothesis that *rte3* loss-of-function mutants affect hypocotyl growth is supported by the double mutant analysis of *rte3-1* with other ethylene receptor alleles. Strong ethylene receptor alleles were not affected by the presence of the *rte3-1* mutation, while weakly insensitive alleles like *etr1-2* and *ers1-10* were affected much more. While *rte3-1 etr1-2* and *rte3-1 ers1-10* etiolated seedlings treated with ACC were much shorter than those not treated with ACC, the *etr1-2* and *ers1-10* mutants also respond to ACC to a small degree. It is conceivable that the hypocotyl growth defect of *rte3* could lead to the appearance of a stronger response to ethylene treatment in these weak ethylene-insensitive alleles. This is supported by the fact that *rte3-1 etr1-2* and *rte3-1 ers1-10* are shorter than the corresponding single mutants even when not treated with ethylene.

rte3 may be specifically regulating seedling hypocotyl growth in the dark. While not directly relating to ethylene signaling, hypocotyl growth has an effect on a phenotype related to an ethylene phenotype, the triple response. This is the most likely reason *rte3* was isolated as an ethylene suppressor mutant in the first place. Therefore, to be able to study the defect in the *rte3* mutant further, a map-based cloning approach was used to clone the *RTE3* gene. Once cloned, a genomic clone of the *RTE3* gene was able to rescue the *rte3* phenotype, demonstrating that the *rte3-1* mutation is responsible for the mutant phenotype. The *RTE3* gene was found to

encode a predicted soluble protein with no known function in *Arabidopsis*. The only conserved domain in the RTE3 protein is a SAC3/GANP domain, which has no known molecular function. Although it has no known function, the SAC3/GANP domain is usually found within proteins that participate in large macromolecular complexes. A few examples include Sac3p in the mRNA export complex in *Saccharomyces cerevisiae*, RPN12 in the regulatory subunit of the 26S proteasome in eukaryotes, and eIF3k in the eIF3 tanslation initiation complex of eukaryotes (Burks et al., 2001; Lei et al., 2003; Seeger et al., 1996; Gordon et al., 1996). This suggests the possibility that RTE3 acts within a large protein complex to regulate seedling growth.

Since both *rte3-1* and the second loss-of-function allele, *rte3-2,* are present near the 3' end of the *RTE3* gene, RT-PCR analysis was performed to determine *RTE3* expression in the *rte3-1* mutant. Although *rte3-1* appears to be knocked-down, it is not knocked-out, showing that *rte3-1* is not a true null allele. Attempts to obtain a true null with a T-DNA insertion were unsuccessful, since one T-DNA allele present within an intron did not have the *rte3* phenotype, and I was unsuccessful in obtaining a homozygous line for other T-DNA insertion lines. It is certainly possible that a true null in *rte3* has a lethal phenotype, but this is not supported by any empirical evidence.

Since no known molecular function is known for RTE3, the sub-cellular localization of an RTE3-GFP fusion protein was determined in order to provide any clues to RTE3's function. An RTE3-GFP fusion protein was localized to the nucleus in light-grown *Arabidopsis* roots, but was expressed at low levels or not at all in dark-

grown *Arabidopsis* roots. This indicates RTE3 is regulated in response to light conditions, but this is opposite of what the phenotype suggests. One would expect to see RTE3-GFP in dark grown plants, since the original *rte3* phenotype was a dark-grown one. However, this inconsistency might be explained by the fact that the *rte3* phenotype is a hypocotyl phenotype, whereas the localization was performed in roots. It is certainly possible that RTE3 could be expressed differentially in separate tissue types, and further localization analysis should be carried out in hypocotyls in light and dark growth conditions. Another possibility is that RTE3 may need to be degraded to be activated. Recent studies have shown that the plant transcription factor NPR1 undergoes cycles of activation and degradation in response to pathogen attack (Spoel et al., 2009). An additional example of a protein that is degraded to promote activation of the protein is the floral development regulator LFY (Chae et al., 2008). If RTE3 undergoes similar regulation, the absence of RTE3 in dark-grown seedlings may actually indicate greater activity. Studies into the degradation of RTE3 by the proteasome are needed to confirm this hypothesis.

The lack of a definitive phenotype for the *rte3* mutant raises the question of what exactly is the RTE3 protein doing? Although clues have been provided by phenotype analysis and protein localization, there is no known molecular function for the RTE3 protein. RTE3 probably has something to do with etiolated seedling growth, but exactly how this effect occurs is unknown. Since RTE3 is localized to the nucleus, RTE3 may be involved in gene regulation or involved in some type of nuclear transport. These are mere guesses however, and do not have any evidence to back them up. Therefore, to gain insight into the function of the RTE3 protein, I

hypothesized that RTE3 could be involved in a large multi-protein complex based on the presence of a SAC3/GANP domain within its structure. This work will be presented in the next chapter.

Materials and Methods

Plant Growth and Conditions - Unless otherwise stated, all *Arabidopsis* plants used in this study are of the Columbia (Col-0) ecotype. *rte3-3* is a T-DNA insertion line (WiscDsLox489-492C8) available from the Arabidopsis Biological Resource Center (ABRC). Other T-DNA insertion lines (SALK_042128, SALK_141523, SALK_098621) were also obtained, but difficulties in genotyping led to the lack of homozygous lines for each of these T-DNA insertion lines. For seedling growth and triple response assays, seeds were plated onto Murashige and Skoog (MS) (Sigma Aldrich) media containing 0.8% agar. Where indicated for ethylene response and ethylene inhibitor assays, plates also contained 100 μ*M* ACC, 100 μ*M* $AgNO_3$, or 10 μ*M* AVG (Sigma Aldrich). Plates were cold stratified at 4°C for three days, followed by growth at 20°C in the dark for four days. For ethylene dose response assays, plates were placed in airtight jars with indicated concentrations of ethylene gas (Airgas), and jars were kept in dark for four days. Seedlings were photographed and hypocotyl lengths were measured using ImageJ software (http://rsbweb.nih.gov/ij/). For soil-grown plants, seeds were either sown directly onto soil and cold-stratified for 3 days at 4°C, or seedlings were transferred from plates. Plants were grown on MetroMix 360 Growing Medium (SunGro Horticulture) under cycles of 16 hours light and 8 hours dark at 22°C in light and 20°C in dark.

Mutagenesis and Mutant Screening – For mutagenesis, 200 mg of seeds of the *etr1-2* genotype were washed in a solution of 0.1% Tween 20 (Sigma Aldrich). Seeds were then incubated in a solution of 0.3% EMS (Sigma Aldrich) for 12 hours with gentle agitation. Seeds were washed twice with water and suspended in 0.1% agarose. Seeds were sown onto soil and allowed to grow to maturity. Seeds were collected from these M_1 plants and plated onto 100 μ*M* ACC plates (described above) for screening. Suppressor mutants were isolated based on the exhibition of the triple response.

Senescence Assays – For adult plant senescence, four-week old plants were placed in airtight chambers with either air or 100 ppm ethylene. After four days of treatment, plants were removed and photographed.

Cell Size Assay - Seedlings were grown in the dark for four days, then placed on wet mounts to be imaged using a Nikon Labphot-2 microscope equipped with a Zeiss Axiocam IcC3 camera. Photos of hypocotyls were taken, and hypocotyl cell lengths were measured using ImageJ software (see above).

Gene mapping and cloning – For mapping, the *rte3-1 etr1-2* mutant was crossed to a Landsberg erecta line carrying a dominant, ethylene-insensitive *etr1* mutation. Plants from the F_2 population of this cross were screened for exhibition of the triple response phenotype. Plants that exhibited the triple response were used for the mapping population. All mapping population individuals had their progeny's triple response assayed to confirm the phenotype in the parent. DNA was isolated from each individual and assayed using markers designed from Monsanto's Ler polymorphism collection (available at: www.arabidopsis.org). For sequencing,

genomic DNA from *rte3-1 etr1-2* was PCR amplified using target specific primers, then sequenced using standard dideoxy (Sanger) reactions.

Plasmids and cloning – To obtain the genomic *RTE3* clone with native promoter, three distinct fragments of the *RTE3* gene were amplified from genomic DNA. N-terminal fragment primers: 5' GGGGACCACTTTGTACAAGAAAGCTGGGTTTC AGAAGTAAATACAGAGCTTCTC 3' and 5' GAGAAACGAAAACATTATGACA CTG 3'. Middle fragment primers: 5' TTCACTCCTTATACCTGTAAAGG 3' and 5' ACAGCAGATTATCATCGTTGAGAC 3'. C-terminal fragment primers: 5' ATCAA CAGTGTATTACCGCCCAT 3' and 5' GGGGACCACTTTGTACAAGAAAGCTG GGTTGAAGTAAATACAGAGCTTCTCG 3'. Each fragment was cloned into the pGEM-T vector (Promega), and the N-terminal and middle fragment plasmids were digested with SalI and AvrII. The 6.4 kb fragment from the N-terminal plasmid and the 4.2 kb fragment from the middle fragment plasmid were ligated to produce a N-terminal plus middle fragment in pGEM-T. The N-terminal plus middle fragment pGEM-T and C-terminal pGEM-T were digested with SalI and the 2.0 kb fragment from the C-terminal plasmid was ligated into the linearized N-terminal plus middle fragment plasmid to produce the full length *RTE3* clone in pGEM-T. This plasmid was used in a BP-reaction with pDONR221 (Invitrogen) to generate an *RTE3* entry clone. This donor plasmid was used in LR clonase (Invitrogen) reactions with pEarleyGate303 (Earley et al., 2006) to generate the myc-tagged RTE3 construct and with pMDC107 (Curtis and Grossniklaus, 2003) to generate the GFP-6xHis-tag RTE3 construct.

RT-PCR – RNA was isolated from *Arabidopsis* seedlings grown for four days under 24-hour light or in the dark at 20° C using the Plant RNeasy Isolation Kit (Qiagen). cDNA was made from this RNA using the iScript Select cDNA Synthesis Kit from Bio-Rad and used in subsequent PCR analysis. For cloning the coding sequence, the following primers were used: 5' GGGGACAAGTTTGTACAAAAAAGCAGGCTCA ATGGCGTTTAGGCCTTTCGG 3' and 5' GGGGACCACTTTGTACAAGAAAGC TGGGTTTCAGAAGTAAATACAGAGCTTCTC 3'. Resulting PCR fragments were used in a BP clonase reaction with pDONR221 (Invitrogen), and the plasmids were sequenced. For overexpression analysis, the *RTE3* coding sequence in pDONR221 was used in an LR clonase (Invitrogen) reaction with pH2GW7 (Karimi et al, 2002) to generate a 35S::*RTE3* expression clone.

Confocal Microscopy – *Arabidopsis* plants were grown at 20° C in darkness or under 24-hour light for four days. Seedlings were incubated in 5 μg/mL DAPI for 20 minutes, then roots were excised and mounted in VectaShield mounting medium (Vector Laboratories). Seedlings were then imaged using a Leica SP5 X confocal microscope.

RTE3 is a member of a multi-protein complex including EIN2, EER5, and a member of the COP9 signalosome

Introduction

In the previous chapter, I provided evidence that *RTE3* is not involved in ethylene responses. *rte3-1 etr1-2* only had a partial triple response phenotype, and when assayed for other ethylene phenotypes, *rte3-1* was unable to suppress *etr1-2* ethylene insensitivity. In addition, *rte3-1* was unable to suppress several other ethylene-insensitive receptor mutants. When the *RTE3* gene was cloned, it was found to encode a protein with a SAC3/GANP domain, a domain present in large protein complexes. The RTE3 protein was found to localize to the nucleus, indicating that it may have a role in gene regulation or nuclear transport.

Since *RTE3* had been determined to not play a role in ethylene signaling, the question of what role the *RTE3* gene plays in *Arabidopsis* needed to be answered. One clue may come from the sole annotated domain within the RTE3 protein, a SAC3/GANP domain. SAC3/GANP domain-containing proteins are found in all eurkaryotes, and the proteins that contain this domain are usually found in large multi-peptide complexes. A few examples of SAC3/GANP domain-containing proteins include Sac3p in *Saccharomyces cerevisiae*, a member of the yeast mRNA export complex, the 26S proteasome regulatory subunit member RPN12, and a member of the eIF3 translation initiation complex, eIF3k (Burks et al., 2001; Lei et al., 2003; Seeger et al., 1996; Gordon et al., 1996).

Especially interesting among these complexes is that they share a similar protein composition. That is, these complexes contain proteins that are homologous to one another. One common motif found among these protein complexes is the Proteasome/COP9/Initiation factor (PCI) domain, which is found in members of the 26S proteasome regulatory subunit and the eIF3 translation initiation complex (Wei and Deng, 2003). Interestingly, the COP9 signalosome (CSN) displays a similar protein composition as the 26S proteasome regulatory subunit and the eIF3 complex. These complexes share the aforementioned PCI proteins and other homologous proteins that contain another type of domain, the MPN domain (Wei and Deng, 2003). The final protein domain shared among these complexes is the PCI-associated module (PAM) domain, which is found in members of the 26S proteasome, the CSN, and the yeast mRNA export complex, but not the eIF3 complex (Ciccarelli et al., 2003).

One feature that the CSN does not have is the presence of a SAC3/GANP-domain containing protein, despite its resemblance to other complexes that do contain SAC3/GANP domain-containing proteins. This leads to the hypothesis that RTE3 may interact with members of the CSN. There is evidence that members of the CSN may participate in other interactions and functions outside of the CSN derubylation complex. CSN5 has been seen in unidentified small protein complexes by gel filtration assays (Gusmaroli et al., 2004; Gusmaroli et al., 2007). This raises the possibility that the proteins of the CSN could interact with other protein complexes to perform diverse functions in the *Arabidopsis* plant.

This has been supported by the finding that a previously uncharacterized PAM domain-containing protein, EER5 interacts with a member of the CSN, a previously uncharacterized interaction (Christians et al., 2008). What makes this data especially interesting is the fact that the *eer5* mutant was initially isolated in a screen for enhanced ethylene response mutants. The *eer5* mutant has an extremely short hypocotyl in response to ethylene treatment, and the hypocotyl is shorter than wild type even when not treated with ethylene (Christians et al., 2008). This phenotype is remarkably similar to the triple response phenotype of *rte3*, although senescence was not assayed in the characterization of *eer5*. Additionally, in the Christians et al. paper, the authors demonstrate that the EER5 protein interacts with the C-terminal portion of EIN2, and that the same C-terminal portion of EIN2 interacts with members of the CSN. This seems to demonstrate that EER5 may be a member of a sub-complex of the CSN that regulates some aspect of ethylene response.

This raises several questions about the role of *RTE3* in *Arabidopsis*. Does *RTE3* interact with members of the CSN, since the protein domains suggest that there may be one? Since the *eer5* and *rte3* mutants have similar phenotypes, what is their relationship to each other? Are they in parallel pathways and do they interact or participate in the same complex? If RTE3 physically interacts with EER5, does RTE3 also interact with EIN2, and if so, how does the lack of an ethylene phenotype in the *rte3* mutant relate with the fact that *EIN2* is a central member of the ethylene signaling pathway? I aimed to answer these questions by studying the physical interactions of the RTE3 protein, by studying the genetic interactions between *RTE3*

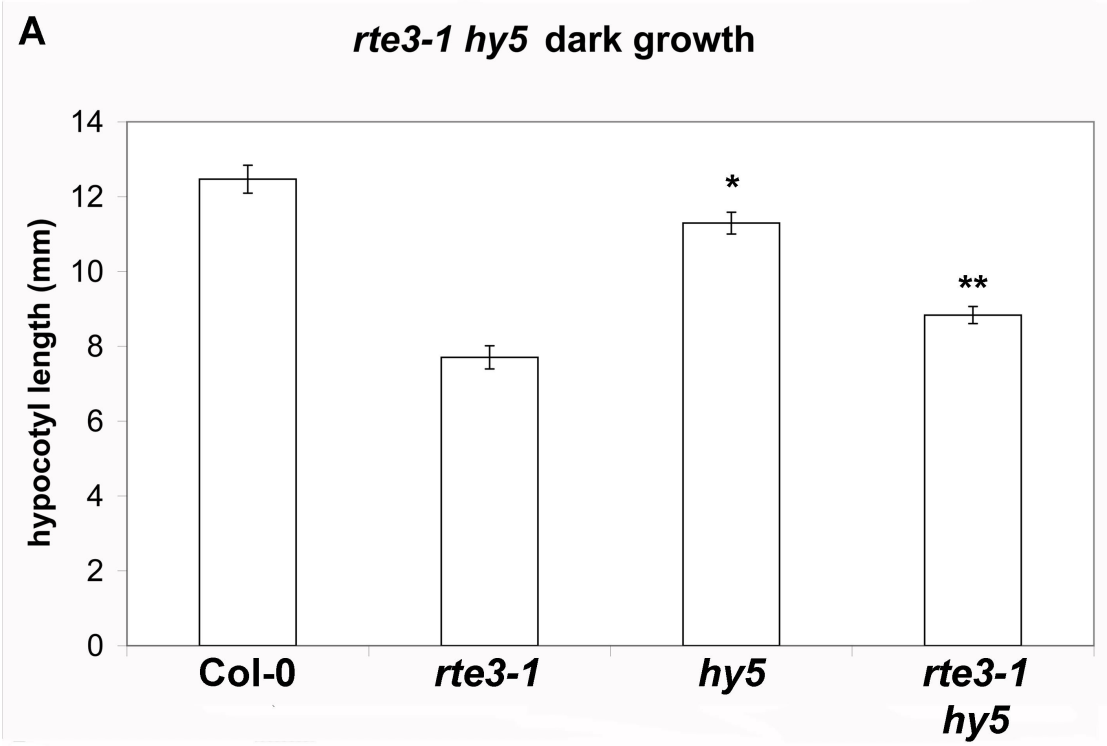
and *EIN2*, and by assaying the *rte3* mutant for typical CSN-related phenotypes. I will present the results of that work in this chapter.

Results

The *rte3* mutant does not display typical *csn* mutant phenotypes.

Since the RTE3 protein may have an interaction with members of the CSN due to the presence of a SAC3/GANP domain, the *rte3* mutant was assayed for phenotypes seen in *csn* mutants. While null alleles of any CSN subunit results in a severe photomorphogenic and seedling lethal phenotype, weaker alleles of the rubylation/derubylation pathway often have auxin insensitive or constitutive photomorphogenic phenotypes (Gusmaroli et al., 2007; Schwechheimer et al., 2001). Therefore, the *rte3-1* mutant was tested for altered light responses under blue, red, far-red, white light, and dark conditions. Under each of these conditions, weak CSN mutants display shorter hypocotyls than wild-type seedlings (Gusmaroli et al., 2007). Therefore, if the RTE3 protein has a role together with any members of the CSN, the *rte3* mutant may display weak constitutive photomorphogenic phenotypes. When grown under each of the above light conditions for five days, the *rte3-1* mutant did not display growth altered from wild type under all light conditions tested (Figure 19B). The only growth defect was seen in the dark, which has been noted before in triple response assays.

Although there were no phenotypes displayed in light conditions, it is still possible that the hypocotyl growth defect in the dark is an extremely weak constitutive photomorphogenic phenotype. To test this possibility, the *rte3-1* mutant was crossed to a *hy5* null mutant. *hy5* is a transcription factor responsible for


A
rte3-1 hy5 dark growth
hypocotyl length (mm)
14
12
10
8
6
4
2
0
*
**
Col-0
rte3-1
hy5
rte3-1
hy5

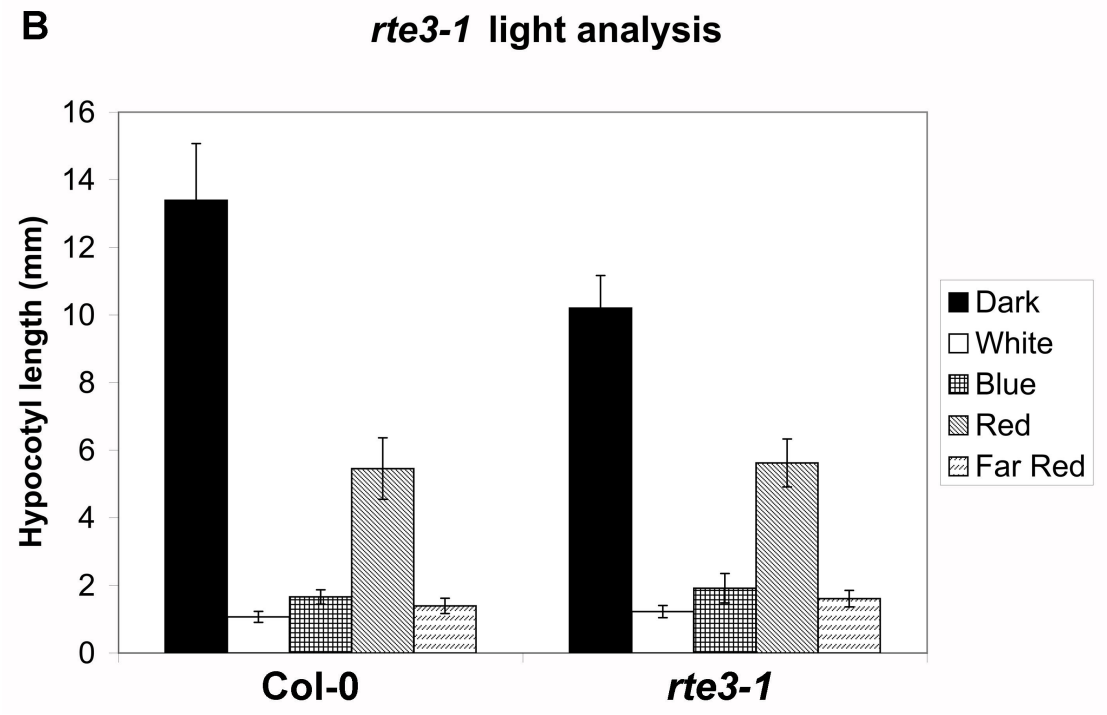

B
rte3-1 light analysis
Hypocotyl length (mm)
16
14
12
10
8
6
4
2
0
Dark
White
Blue
Red
Far Red
Col-0
rte3-1

Figure 19 – The *rte3* phenotype is not a constitutive photomorphogenesis phenotype. A) Etiolated seedlings were grown on MS plates for four days. Seedlings were photographed and hypocotyls measured. Asterisks represent statistically significant difference between *rte3-1* and *rte3-1 hy5* data points (Student's t-test: $p<5*10^{-8}$). Error bars represent standard error of the mean. Data represents one experiment where n = 20 for each data point. B) Seedlings were grown on MS plates with the indicated light treatments for five days. Seedlings were photographed and measured. Error bars represent standard deviation. Data represents one experiment where n = 20 for each data point.

induction of light-responsive growth and is degraded in the dark, a process that is dependent upon the ubiquitin ligase COP1 (Koornneef et al., 1980; Osterlund et al., 2000). This process is regulated by the COP9 signalosome, and so the constitutive morphogenesis phenotype of any *csn* mutant is mediated through *HY5* (Osterlund et al., 1999). Therefore, if the *rte3-1* phenotype is a photomorphogenic phenotype associated with the CSN, then introducing a *hy5* null mutation should suppress that phenotype. When the *hy5* null mutant was crossed with the *rte3-1* mutant, the double mutant showed the same hypocotyl growth defect in the dark as the *rte3-1* mutant (Fig. 19A). Therefore, it is unlikely that *RTE3* has a role in photomorphogenesis.

Another phenotype that *csn* mutants often display is an auxin insensitive phenotype (Schwechheimer et al., 2001). When treated with low doses of the auxin analog 2,4-dichlorophenoxyacetic acid (2,4-D), a weak *csn* mutant will display insensitivity to that treatment (Zhang et al., 2008). The *rte3-1* mutant was tested for its response to 50 n*M* 2,4-D in a root growth assay (Fig. 20). When compared with wild type, the *rte3-1* mutant has shorter roots even without auxin treatment. With treatment, the rate of inhibition for *rte3-1* is similar to wild type, indicating that the *rte3* mutant does not have an auxin insensitive phenotype. However, *rte3* roots grown in light are shorter overall. This also explains the expression of RTE3-GFP in light-grown roots (Fig 16). If *RTE3* is involved in root growth, its presence in root nuclei is not a mystery.

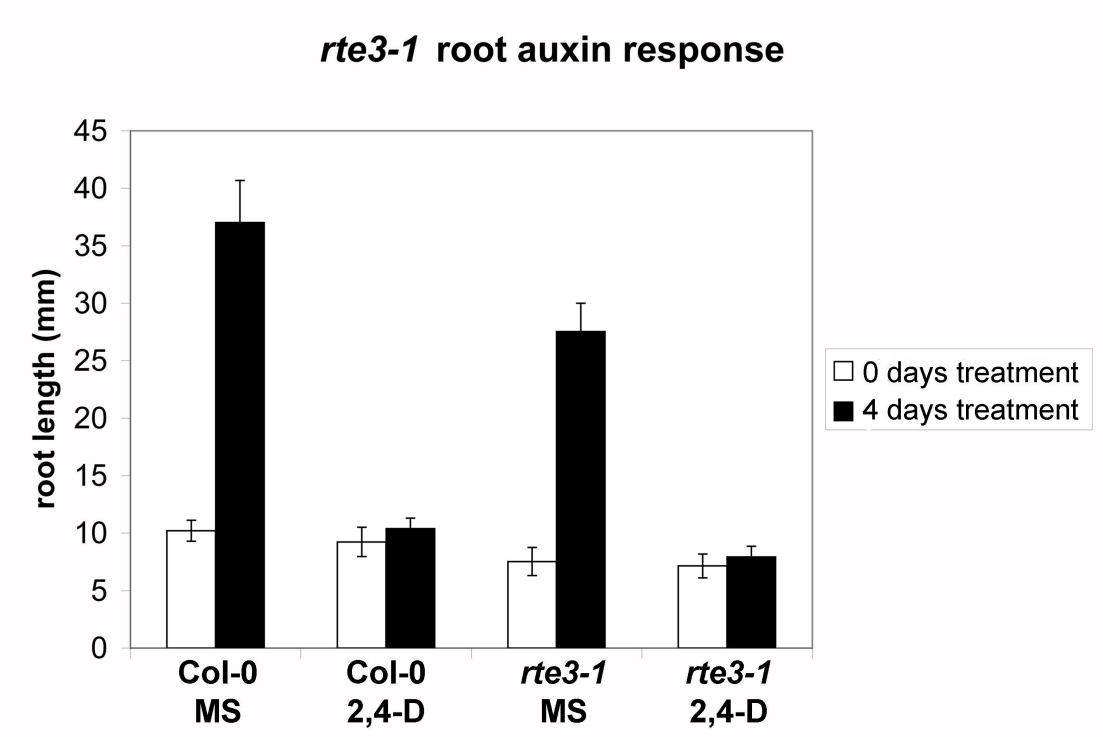


Figure 20 – The *rte3-1* mutant does not display an auxin-insensitive phenotype, but shows a light-grown root length phenotype. Seedlings were grown on vertical MS plates for four days under 24-hour light, then transferred to either vertical MS plates or vertical MS plus 50 n*M* 2,4-D plates for four days. Seedlings were photographed and root lengths measured. Error bars represent standard deviation. Data represents one experiment where n = 10 roots.

RTE3, EER5, CSN3, and EIN2 C-terminus interact in a yeast two-hybrid screen

Since only a general seedling growth defect had been noted for the *rte3* mutant, an attempt to gain insight into its molecular function through yeast two-hybrid screening was made. Proteins containing a SAC3/GANP domain seem to be present in large, multi-peptide complexes, so a yeast two-hybrid approach to protein interactions was undertaken. Previously, the EER5 protein, a PAM domain-containing protein, was found to interact with both members of the CSN and the C-terminus of EIN2 (Christians et al., 2008). Since the PAM domain is a motif common amongst proteins within the CSN, 26S proteasome, and the Sac3 mRNA export complex, RTE3 was used in a yeast two-hybrid assay to test for physical interaction with EER5, EIN2, and CSN3, a member of the COP9 signalosome. The EER5 and CSN3 full length coding sequences and the C-terminus (amino acids 516-1294) of EIN2 were cloned into the pACTII vector (Clark et al., 1998). When assayed for interaction with RTE3, each of these clones demonstrated physical interacation with the RTE3 protein (Figure 21).

To possibly confirm these interactions and to find additional interactors of RTE3, a cDNA library screen was performed. The cDNA library was made by Kim et al. (1997) from the RNA of three-day old etiolated seedlings. For the screen, the *RTE3* cDNA was cloned into the pLexA-NLS vector (Clark et al., 1998). Out of $1.8*10^5$ colonies screened, 19 putative interactors were found. Upon re-screening, only one clone still grew on selection media (Fig. 22). Sequencing revealed that the prey vector contained a cDNA derived from At4g27280, designated here as EF HAND1 (EFH1), an annotated EF-hand calcium-binding protein. This is an

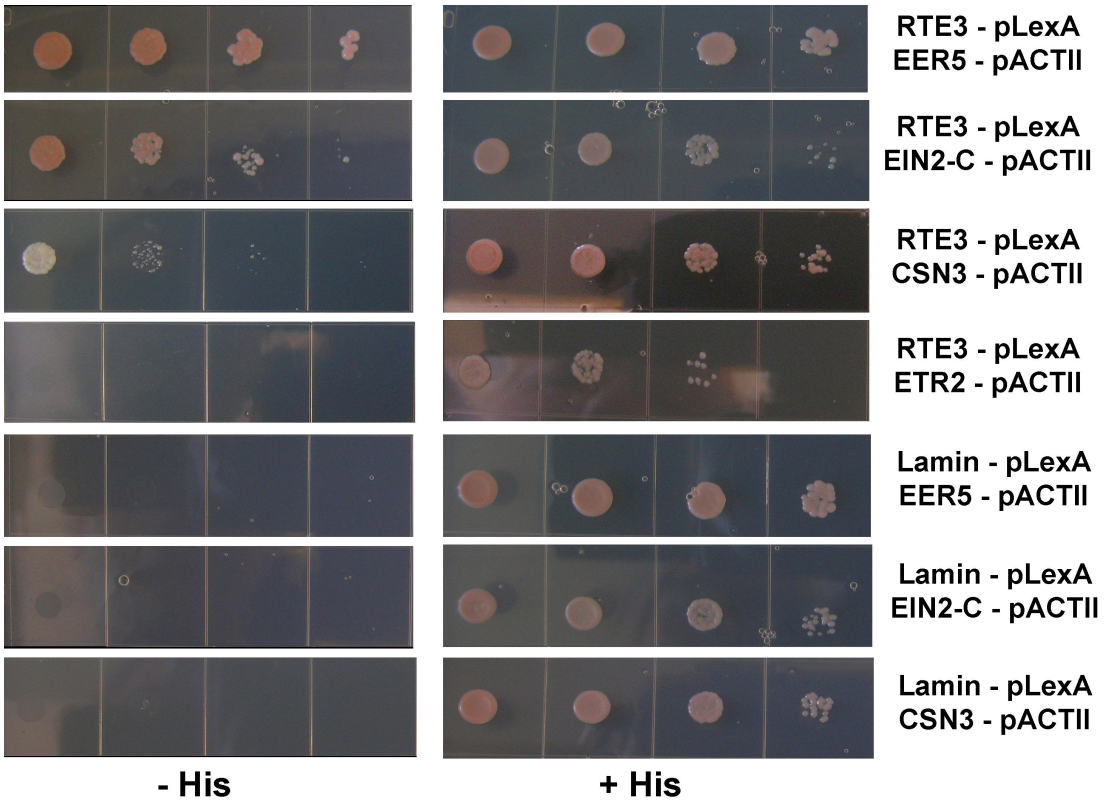


Figure 21 – The RTE3 protein interacts with EER5, CSN3, and the C-terminus of EIN2 in a yeast two-hybrid assay. The *EIN2-C* cDNA (amino acids 516-1294), *EER5*, and *CSN3* cDNAs were cloned into the pACTII prey vector and used with the *RTE3* cDNA in pLexA-NLS vector in yeast two-hybrid analysis. Colonies from non-selective plates were suspended in water and serial dilutions made. Dilutions were spotted on non-selective (SD- Trp, Leu) and selective (SD -Trp, Leu, His, + 20 m*M* 3-AT) plates. Non-selective plates were incubated for two days, while selective plates were grown for four days, both at 30° C. Plates were then photographed. ETR2-pACTII and Lamin-pLexA represent negative controls.

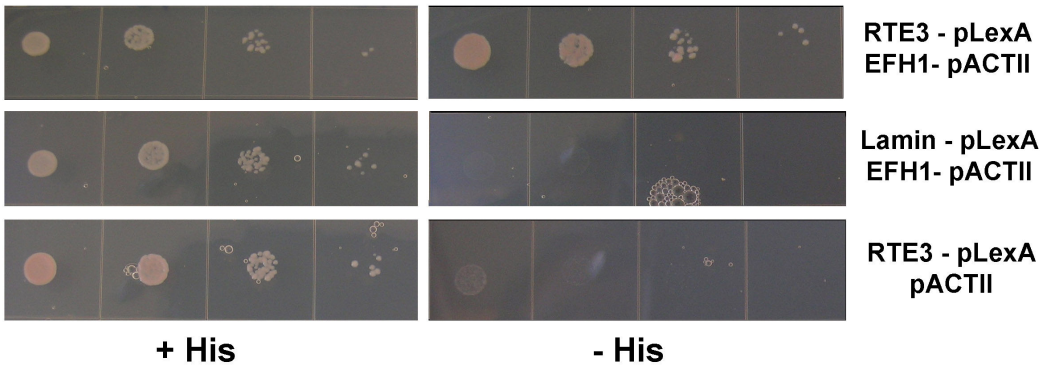


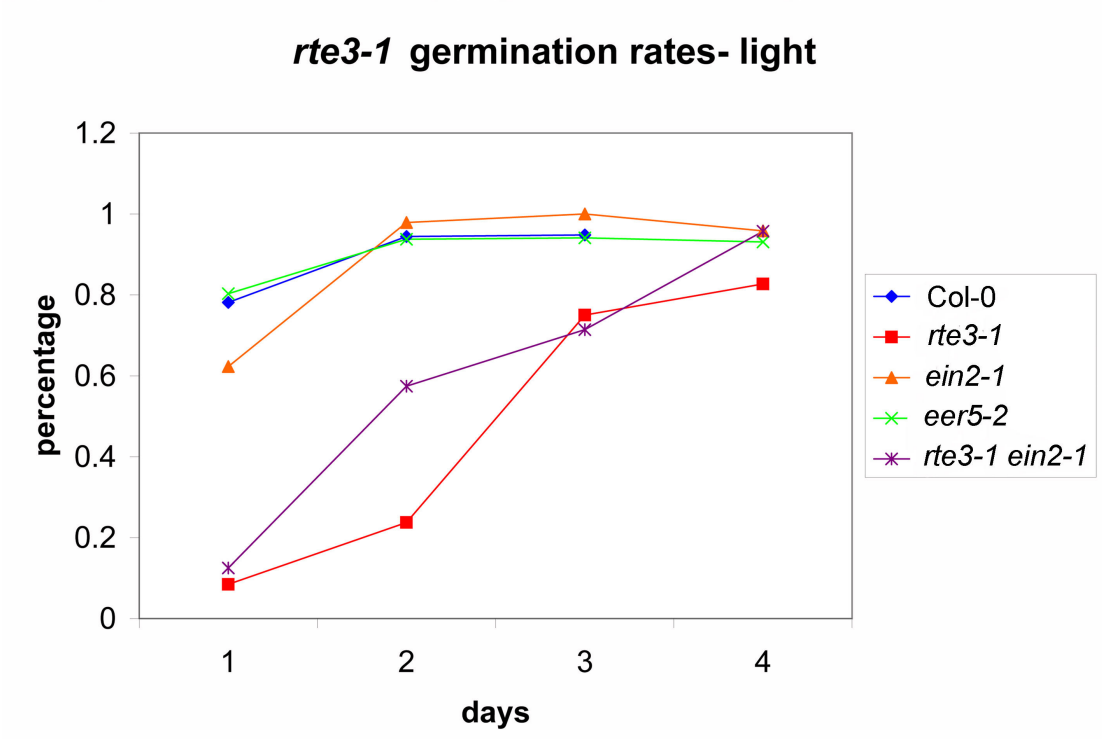
Figure 22 – The EFH1 protein was identified as an interactor of RTE3. The *RTE3* cDNA was cloned into the pLexA-NLS bait plasmid and used to screen the Kim and Theologis λACT library of *Arabidopsis* cDNAs (Kim et al., 1997). Out of $1.8*10^5$ colonies screened, EFH is the only confirmed interacting protein. Yeast colonies from a non-selective medium were suspended in water and serial dilutions spotted on non-selective (SD –Trp, Leu) and selective (SD – Trp, Leu, His + 20m*M* 3-amidotriazole (3-AT)) plates. Yeast plates were incubated at 30° C for two days for non-selective plates and for four days for selective plates, then photographs taken. Lamin – pLexA is negative control.

interesting interactor, since Sac3p in yeast has been shown to be a scaffolding protein and binds an EF-hand motif-containing protein, Cdc31p (Jani et al., 2009). The interaction of EFH1 with RTE3 seems to be a correlation of this interaction, although confirmation must be performed *in vivo*. Although no confirmation of the EER5, EIN2, or CSN3 interactions were found in the library screen, they cannot be ruled out since the screen yielded only one positive clone.

The *rte3-1 ein2-1* double mutant has a delayed growth phenotype

To address the possibility of a false yeast two-hybrid interaction, the genetic interaction of the *RTE3* and *EIN2* genes were studied. To accomplish this, the *rte3-1* and *ein2-1* mutants were crossed and the double mutant isolated. Initially, I attempted to assay the triple response phenotype of the double mutant, but poor germination in the double mutant defeated this assay. After re-growing the double mutant seed stocks three times, seed germination remained a problem for each seed stock. This was perhaps a new phenotype to quantify, so the germination rate, as determined by the rupture of the seed coat, was measured in the *rte3-1 ein2-1* double mutant. I found that both the *rte3-1 ein2-1* double mutant and the *rte3-1* single mutant have delayed germination rates in both light and dark conditions (Fig. 23). Even though the *rte3-1* mutant has a similar germination rate as *rte3-1 ein2-1*, the double mutant has more seedlings that fail to grow after germination.

To resolve the differences between the *rte3-1* single mutant and the *rte3-1 ein2-1* double mutant, development was observed over the first six days of germination. I found that while the germination rates are similar for both the single and double mutants, *rte3-1 ein2-1* seedlings develop much more slowly over the first


rte3-1 germination rates- light
1.2
1
0.8
0.6
0.4
0.2
0
percentage
1
2
3
4
days
Col-0
rte3-1
ein2-1
eer5-2
rte3-1 ein2-1

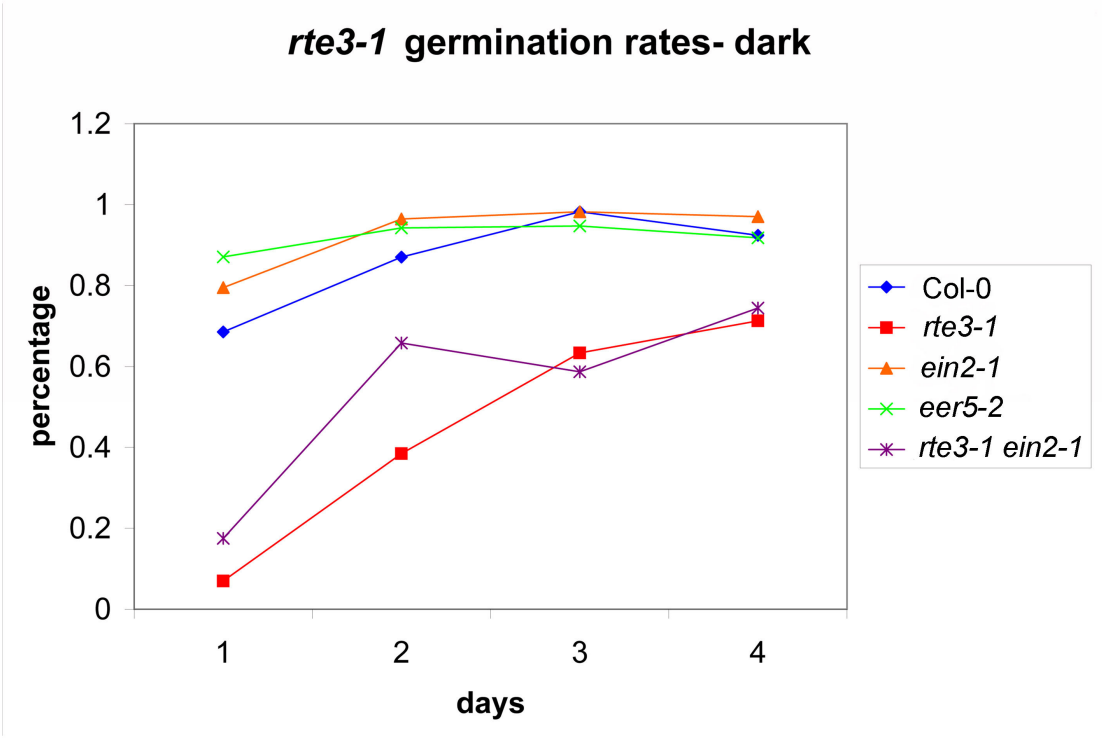

rte3-1 germination rates- dark
1.2
1
0.8
0.6
0.4
0.2
0
percentage
1
2
3
4
days
Col-0
rte3-1
ein2-1
eer5-2
rte3-1 ein2-1

Figure 23 – Both *rte3-1* and *rte3-1 ein2-1* have delayed germination rates in both light and dark conditions. A) Seeds were sown on MS plates and grown under 24-hour light at 20°C for the time indicated. After incubation, seeds were analyzed for rupture of the seed coat as a measure of germination rate. ($40<n<80$ for each data point) B) Seeds were sown on MS plates and grown in the dark at 20° C for the time indicated. After incubation, seeds were analyzed with a Zeiss Stemi 2000-C dissecting microscope for rupture of the seed coat as a measure of germination rate. Data represents one experiment where $40<n<80$ for each data point.

four days of development (Fig. 24). This is perhaps the reason very few suitable seedlings for a triple response assay could be obtained for the *rte3-1 ein2-1* double mutant. Despite it not being a germination defect, there is a synergistic effect between the *rte3* and *ein2* mutants.

The *rte3-1 ein2-1* mutant could display a possible connection to the CSN because *csn* knock-out mutants display seedling lethality that is attributed to genomic instability and cell cycle arrest (Dohmann et al., 2008). One possible reason for the delayed growth seen in the *rte3-1 ein2-1* double mutant could be that the cell cycle is disrupted in these mutants. One hallmark of the *csn* mutants in terms of cell cycle arrest is that cell cycle marker genes are generally upregulated in these mutants.

So, to assay whether the cell cycle is being altered in the *rte3-1 ein2-1* mutant, I performed RT-PCR and qRT-PCR for several cell cycle marker genes in the double mutant. Cell cycle markers were selected based upon microarray data that specified cell cycle stage-specific expression patterns (Menges et al., 2005). RT-PCR analysis was performed using RNA isolated from four-day old seedlings grown in light and dark conditions. Analysis of the resulting gels indicated that several genes in the *rte3-1 ein2-1* double mutant may be upregulated (Fig. 25A).

However, to conclusively say whether these genes are upregulated in the double mutant, quantitative RT-PCR (qRT-PCR) analysis was performed. For qRT-PCR analysis, a subset of markers was chosen: *CYCB1;1*, a G2/M phase transition marker, *PARP1*, a DNA damage marker, and *CYCA3;2*, a S phase marker. *CYCB1;1* and *PARP1* were selected because they show high induction in *csn* mutants (Dohmann et al., 2008). *CYCA3;2* also shows induction in *csn* mutants, and showed

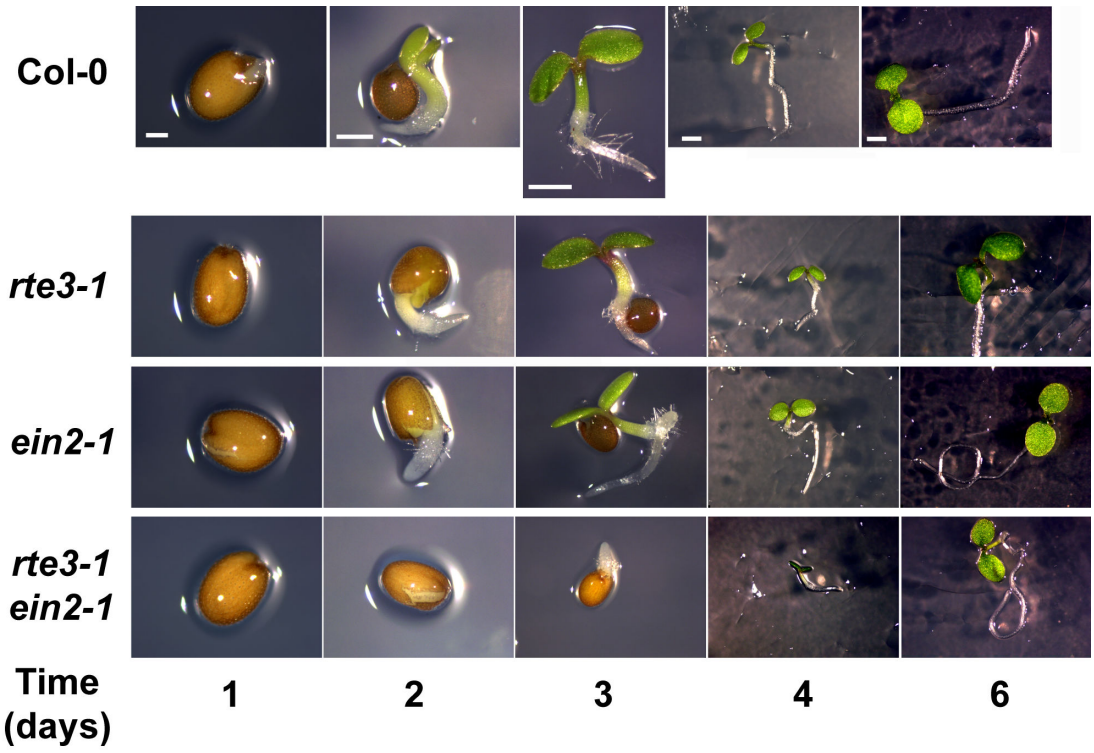


Figure 24 – The *rte3-1 ein2-1* displays a delayed growth phenotype. Seeds of the indicated genotypes were sown on MS plates and grown under 24-hour light for the indicated number of days. After incubation, representative seedlings were imaged using a Zeiss AxioCam ICc3. Scale bar at one day represents 0.2 mm. Scale bar at two days represents 0.5 mm. Scale bars at three, four, and six days represent 1 mm.

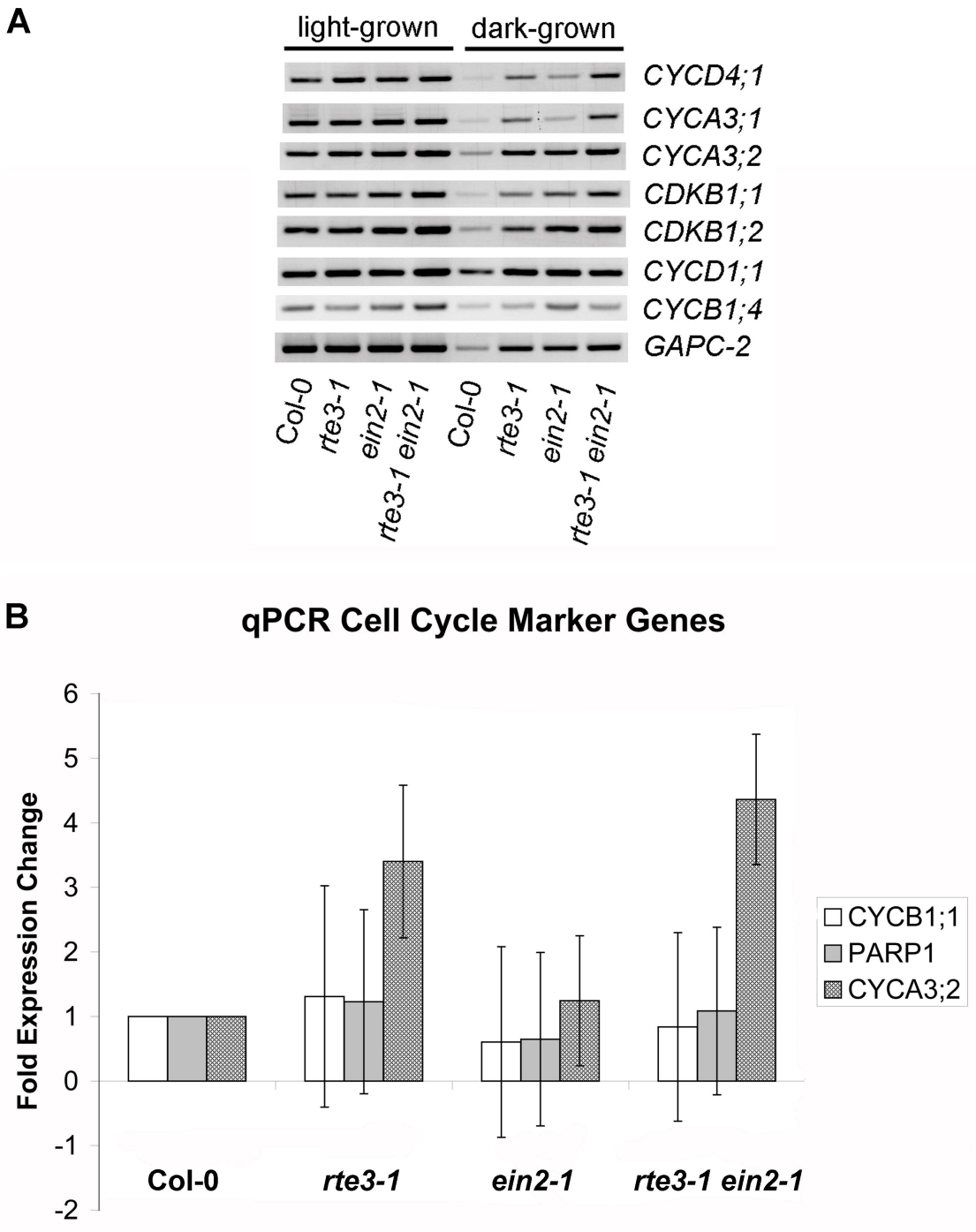


Figure 25 - RT-PCR analysis of cell cycle marker genes shows no correlation between the *rte3-1 ein2-1* phenotypes and the *csn* phenotypes. A) RNA was isolated from four-day old seedlings of each of the indicated genotypes. Plants were either grown under 24-hour light or in the dark. cDNA was made from isolated RNA and

used in PCR analysis. B) RNA isolated from four-day old *Arabidopsis* seedlings grown under 24-hour light was used to make cDNA. cDNA was used in qRT-PCR analysis using the Light Cycler 480 SYBR Green I master kit (Roche). Three technical replicates per sample and three biological replicates per condition were used in the analysis. Reactions were run using a Light Cycler 480 Real Time PCR machine (Roche). Values are fold-increases compared to the wild-type sample. Error bars are standard deviations for the three biological replicates.

consistent up-regulation in preliminary studies. When qRT-PCR analysis was performed with three biological and three technical replicates, only *CYCA3;2* showed significant induction (Fig. 25B). This likely rules out the *rte3-1 ein2-1* mutant phenotype as being a canonical *csn* phenotype, since the induction of *CYCB1;1* and *PARP1* were quite distinct in *csn* mutants. Rather, this may indicate a new role for the CSN/EIN2/RTE3 proteins in *Arabidopsis*.

RTE3 and EER5 localize to the nucleus in onion epidermal cells

In order to confirm that the interaction between the RTE3 and EER5 proteins could occur *in planta*, the sub-cellular localization of the EER5 protein in onion epidermal cells was determined. The *RTE3* and *EER5* coding sequences were cloned into pEarleyGate103 (Earley et al., 2006). This expression vector contains a 35S promoter element with a C-terminal GFP tag. The resulting vector was then transiently transformed into onion epidermal cells via particle bombardment. After incubating in the dark for 24 hours, epidermal peels were stained with DAPI and imaged with a fluorescence microscope.

When localized with DAPI, the RTE3-GFP fusion protein localized to the nucleus, consistent with results from *Arabidopsis* roots (Fig. 26). EER5-GFP also localized to the nucleus when co-localized with the DAPI stain, confirming that EER5 and RTE3 localize to the same sub-cellular compartment (Fig. 27). An untransformed control did not have any significant signal (Fig. 28). This localization pattern shows that the yeast two-hybrid interaction between RTE3 and EER5 could occur *in planta*, although *in vivo* confirmation still needs to be performed. In

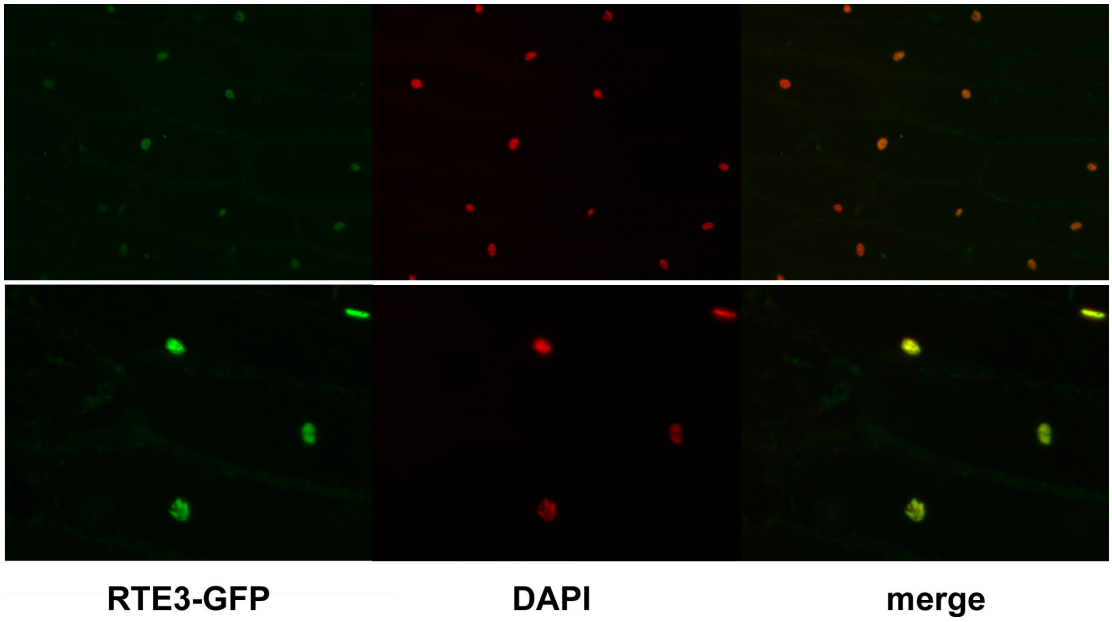


Figure 26 - A RTE3-GFP fusion protein localizes to the nucleus in onion epidermal cells. The *RTE3* coding sequence was cloned into pEarleyGate103, which was then transiently transformed into onion epidermal cells via particle bombardment. After 24 hours of incubation in the dark, epidermal peels were incubated in a solution of 5 μg/mL DAPI for 20 minutes, then imaged using a Zeiss AxioObserver.Z1 fluorescence microscope.

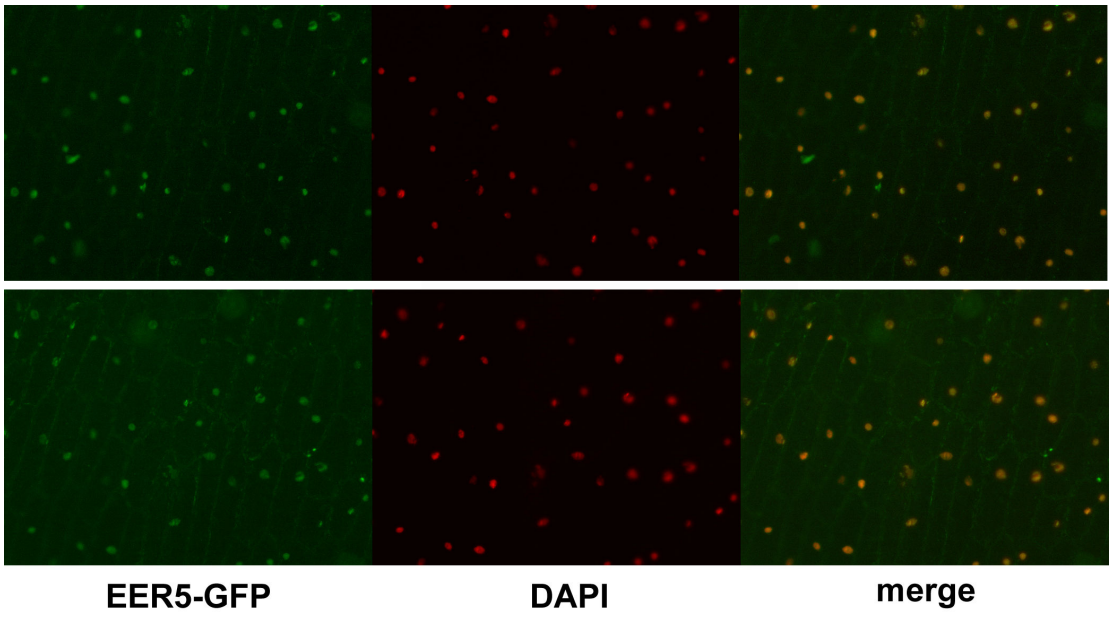


Figure 27 - An EER5-GFP fusion protein localizes to the nucleus in onion epidermal cells. The *EER5* coding sequence was cloned into pEarleyGate103, which was then transiently transformed into onion epidermal cells via particle bombardment. After 24 hours of incubation in the dark, epidermal peels were incubated in a solution of 5 μg/mL DAPI for 20 minutes, then imaged using a Zeiss AxioObserver.Z1 fluorescence microscope.

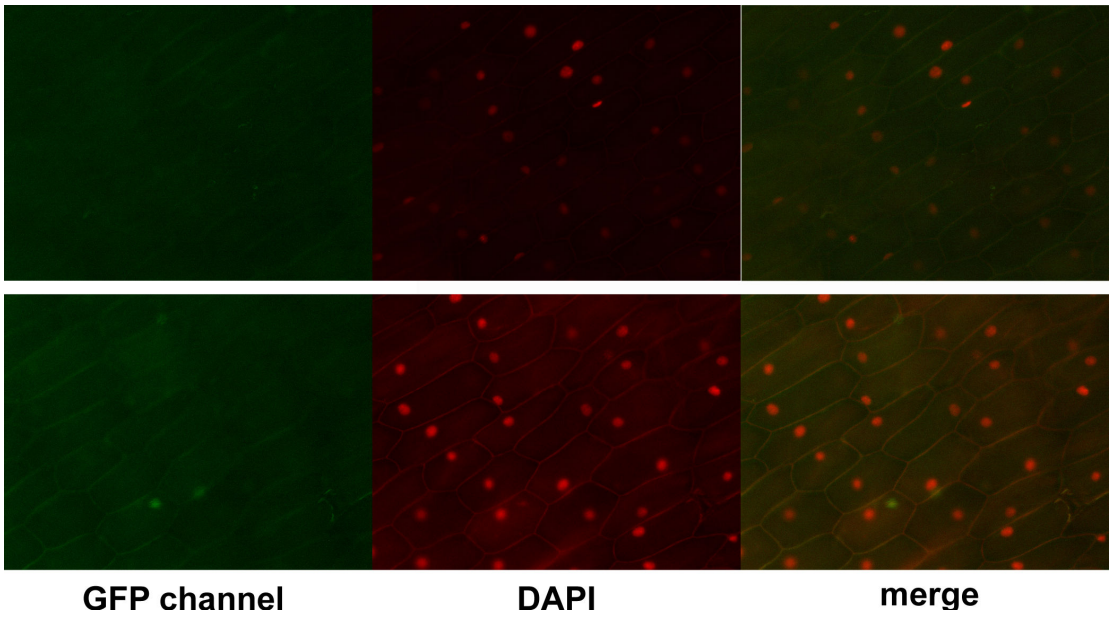


Figure 28 - Untrasnformed control peels do not express GFP. Untransformed onion epidermal peels were incubated in a solution of 5 μg/mL DAPI, then imaged on a Zeiss AxioObserver.Z1 fluorescence microscope.

addition, recent unpublished data suggests that the C-terminus of EIN2 may localize to the nucleus (Ecker, 2009).

Discussion

The CSN, the 26S proteasome regulatory subunit, and the eIF3 complex all have proteins that share common domains. They all share proteins that contain PCI domains, and the CSN and 26S regulatory subunit both contain proteins with PAM domains. However, while the 26S proteasome regulatory subunit and the eIF3 complex contain proteins with a SAC3/GANP domain, the CSN does not have such a protein. Therefore, it was hypothesized that RTE3 may interact with the CSN. However, when phenotypes often seen in *csn* mutants were assayed in the *rte3* mutant, none of the defects were displayed, including auxin-insensitivity and constitutive phtotomorphogenesis. The lack of a hypocotyl growth defect in different light conditions indicates that the *rte3* phenotype is probably not a *csn* defect. This was confirmed by the result that *hy5* is unable to suppress the dark-grown hypocotyl growth phenotype of *rte3-1*. However, it is still possible that RTE3 interacts with members of the CSN, since CSN members may perform different functions or target different proteins separate from that of the CSN complex.

In fact, when tested by yeast two-hybrid analysis, a member of the CSN (CSN3) did interact with RTE3. Additionally, RTE3 interacted with EER5, a PAM domain-containing protein implicated in enhanced ethylene response (Christians et al., 2008), and the C-terminus of EIN2, a central member of the ethylene signaling pathway. Christians et al. (2008) also showed that EER5, the C-terminus of EIN2, and several members of the CSN physically associate with each other. Presuming the

interactions are real, a complex of these proteins appears to regulate some aspects of seedling growth.

These interactions indicate that RTE3 may form a multi-protein complex with EIN2, EER5, and the CSN. However, there is no *in vivo* evidence for these interactions, and confirmations are pending. If confirmed, it would indicate that these proteins act in a complex to regulate seedling growth. Since the activity of the CSN is to modify the activity of the CRL complexes, it is possible that this effect occurs through the modification of one or multiple CRL complexes. Especially interesting is the interaction with the C-terminus of EIN2 and RTE3. Although that *RTE3* does not appear to have an ethylene-response phenotype, EIN2 is a central member of the ethylene signaling pathway. It is certainly possible that EIN2 could have roles outside of ethylene signaling, but a false yeast two-hybrid interaction could also be a possibility.

The only phenotypes demonstrated so far for the *rte3-1* single mutant are a reduction in hypocotyl growth in etiolated seedlings, a reduction in root growth in light-grown seedlings, and delayed germination. In fact, the hypocotyl and root growth phenotypes are most likely a result of the delayed germination. The lack of an ethylene phenotype for *rte3* may indicate that EIN2 and EER5 perform other functions outside of their ethylene response functions, perhaps to regulate cell growth or division. This is partially supported by the fact that *rte3-1 ein2-1* mutant seedlings have a slower development than the corresponding single mutants. Although not lethal, the phenotype exhibited by the double mutant temporarily resembles that of a *csn* knock-out, as the seedling is small and stunted. However, this resemblance does

not last, as double mutant seedlings recover from their developmental delay and grow into healthy adult plants (data not shown). This could indicate that a complex of RTE3, EER5, EIN2, and members of the CSN help regulate cell growth and division. It is not the whole story though, since some members, like the CSN proteins, have more severe phenotypes in relation to seedling growth. It is possible that RTE3, EER5, and EIN2 are modifiers of CSN activity, while the CSN performs the majority of the regulatory activity.

Quantitative PCR of several cell cycle marker genes indicates that the cell division defect present in *csn* mutants is not identical to the defect in the *rte3-1 ein2-1* mutant. While levels of the G2/M cyclin gene *CYCB1;1* and the DNA damage marker gene *PARP1* are induced in *csn* mutants, their levels are unchanged in *rte3-1 ein2-1* mutants. However, the S-phase cyclin gene *CYCA3;2* is upregulated in both *rte3-1* and *rte3-1 ein2-1*mutants. This suggests that *RTE3* has a role in cell cycle regulation, but the exact nature of this role or its consequences are unknown. Exactly why the *rte3-1 ein2-1* has a more severe developmental delay than *rte3-1* is unclear.

In addition to the genetic interaction between *RTE3* and *EIN2* supporting their proteins' physical interaction, the localization of the EER5 and RTE3 proteins to the nucleus support their physical interaction. Since the sub-cellular localization of EIN2 is unknown, it is unclear whether EIN2 would be present in the nucleus to participate in a protein complex with the CSN. EIN2 contains twelve transmembrane domains, so would be unlikely to participate in a nuclear complex. However, unpublished data from Ecker (2009) suggested that the C-terminus of EIN2 is present in the nucleus when *Arabidopsis* plants are treated with ethylene, promoting the hypothesis that

RTE3, EER5, EIN2, and the CSN could form a protein complex. Since the CSN components are enriched in the nucleus, this promotes the model that all of these proteins form a protein complex involved in regulating cell growth and/or division.

The variety of phenotypes exhibited by *csn* and other rubylation pathway mutants suggests that the CSN complex is involved in many different processes within the plant cell. The fact that *rte3* mutants do not display any auxin or light response phenotypes, suggests that the RTE3 complex, consisting of RTE3, EER5, EIN2, and the CSN, is probably not involved in any of these responses mediated by the CSN. Instead, it appears that the complex consisting of RTE3, EER5, EIN2, and the CSN may be involved in regulating another, separate response involved in hypocotyl elongation in the dark and root growth in the light.

Materials and Methods

Plant Growth and Conditions - Unless otherwise stated, all *Arabidopsis* plants used in this study are of the Columbia (Col-0) ecotype. For seedling growth and triple response assays, seeds were plated onto Murashige and Skoog (MS) (Sigma Aldrich) media containing 0.8% agar. Where indicated for ethylene response and auxin response assays, plates also contained 100 μ*M* ACC or 50 n*M* 2,4-D (Sigma Aldrich). Plates were cold stratified at 4°C for three days, followed by growth at 20°C in the dark for four days for triple response assays. Seedlings were photographed and hypocotyl lengths were measured using ImageJ software (http://rsbweb.nih.gov/ij/). For soil-grown plants, seeds were either sown directly onto soil and cold-stratified for 3 days at 4°C, or seedlings were transferred from MS agar plates. Plants were grown on MetroMix 360 Growing Medium (SunGro Horticulture) under cycles of 16 hours

light and 8 hours dark at 22°C in light and 20°C in dark. For light assays, seeds were grown under specified light conditions for five days, then photographed ans measured. Light condition fluency rates: Red (600 nm - 700 nm) = 12.4 μmol/m^2/s; Blue (400-500 nm) = 15.8 μmol/m^2/s; Far-red (700-800 nm) = 26.1 μmol/m^2/s; White = 50 μmol/m^2/s. For root growth assays, seedlings were grown on vertical MS plates for four days under 24-hour light, then transferred to treatment (50 n*M* 2,4-D) or no treatment (MS) vertical plates and incubated under 24-hour light for four more days. Plates were photographed and roots were measured. For seed germination assays, seeds were cold stratified for three days at 4°C, then moved to 24-hour light or dark at 20°C for the time periods indicated. Seed germination was assayed be visible rupture of the seed coat.

Yeast Two-Hybrid Screens - For yeast two-hybrid analysis, all cDNAs used in PCR amplification were made from RNA isolated from four-day old seedlings grown under 24-hour light at 20°C using the Plant RNeasy Isolation Kit (Qiagen). cDNAs were made using the Bio-Rad iScript Select cDNA Synthesis Kit. The *RTE3* coding sequence was amplified from cDNA with the primers: 5' CCCGGGAATGGCGTTTA GGCCTTTCG 3' and 5' CCCGGGTCAGAAGTAAATACAGAGCTTCTCGG 3' and cloned into the pGEM-T Easy vector (Promega). The *RTE3* pGEM-T Easy plasmid and pLexA-NLS (pBTM116) (Clark et al., 1998) were digested with XmaI, and the *RTE3* fragment ligated into pLexA-NLS. The *EER5* coding sequence was amplified from cDNA with the primers 5' GAATTCAAATGGCGTACGTTAGTATGG 3' and 5' CTCGAGTTATGAGCTAACAGGCTTCC 3'. The *EIN2* C-terminal fragment was amplified from cDNA with the primers 5' CCATGGAAGATACTACGTCTGTTACT

AGC 3' and 5' CCCGGGTCAACCCAATGATCCGTAC 3'. The *CSN3* coding sequence was amplified from cDNA with the primers 5' GGATCCAAATGATCGGA GCTGTGAACTC 3' and 5' GAATTCTTACATGGAGAACTTCTGAGG 3'. All three products were cloned into the pGEM-T Easy vector. The *EER5* coding sequence was cloned into pACTII (Clark et al., 1998) using EcoRI and XhoI enzymes, *EIN2* C-terminal sequence using NcoI and XmaI, and the *CSN3* coding sequence using BamHI and EcoRI. All plasmids were transformed into the yeast strain L40. Yeast two-hybrid interaction was performed on -His plates plus 20 m*M* 3-AT. For selection assays, serial dilutions from yeast colonies grown on non-selective media were plated onto selective media and grown at 30°C for four days. For the library screen, the Kim and Theologis *Arabidopsis* etiolated seedling cDNA library in λACT (Kim et al., 1997) (ordered from ABRC) was excised into plasmid form using the protocols developed in Durfee et al. (1993). Briefly, 10^8 library containing bacteriophage were incubated with $3*10^8$ BNN132 cells. These cells were spread onto LB plus 50 μg/mL ampicillin plates and incubated overnight. The resulting lawn of cells (containing the pACT cDNA library) were used to inoculate 1.5 L of TB plus 50 μg/mL ampicillin, which was incubated overnight and used to isolate the cDNA library. Excision efficiency was 20%. For library tranformation, the L40 strain of *S. cerevesiae* carrying the *RTE3*-pLexA-NLS plasmid was transformed in two separate reactions. Both were transformed with 10 μg of library DNA. The first reaction gave $6*10^4$ colonies, while the second gave $1.2*10^5$ colonies, for an overall efficiency of $9*10^3$ cfu/μg of library. Tranformations were plated onto selective plates (-His) plus 20 m*M* 3-AT and grown at 30°C for 14 days. Interacting colonies

were isolated throughout the incubation period, then re-assayed under the same conditions. After these two rounds of interacton selections, plasmid DNA was isolated from potential interactors and transformed into the *E. coli* strain HB101 and plated onto -Leu selection plates to isolate pACT plasmids. Isolated pACT plasmids were re-transformed into L40 plus *RTE3*-pLexA-NLS and assayed for growth on interaction selection plates to confirm putative interactors. The only confirmed interactor was sequenced to determine insert identity.

RT-PCR and qRT-PCR - RNA was isolated from *Arabidopsis* seedlings grown for four days under 24-hour light or in the dark at 20° C using the Plant RNeasy Isolation Kit (Qiagen). cDNA was made from this RNA using the iScript Select cDNA Synthesis Kit from Bio-Rad. cDNA was used in subsequent PCR reactions for RT-PCR gel analysis and quantitative RT-PCR (qRT-PCR) analysis. For primers used see Table 1. qRT-PCR was performed using the Light Cycler 480 SYBR Green I Master Kit (Roche) on a Light Cycler 480 Real Time PCR machine (Roche). For qRT-PCR analysis, three biological replicates were isolated for each genotype tested. For each qRT-PCR reaction, three technical replicates were performed.

Onion Epidermal Cell Localization - The *RTE3* and *EER5* coding sequences were amplified from cDNA (see above). Primers used were: *RTE3*: 5' GGGGACAAGTT TGTACAAAAAAGCAGGCTCAATGGCGTTTAGGCCTTTCGG 3' and 5' GGGG ACCACTTTGTACAAGAAAGCTGGGTTGAAGTAAATACAGAGCTTCTCG 3' *EER5*: 5' GGGGACAAGTTTGTACAAAAAAGCAGGCTTTATGGCGTACGTTA GTATGGGTGAAG 3' and 5' GGGGACCACTTTGTACAAGAAAGCTGGGTTTG AGCTAACAGGCTTCCCGTTTAG 3'. These coding sequences were each cloned

RTE3 For	5' AGGATGGGGTCATCTTAAATC 3'
RTE3 Rev	5' CAATGTTAAACTCACCACACG 3'
CYCD4;1 For	5' TACACCAGTTTGGACCATTG 3'
CYCD4;1 Rev	5' AAGCTCCATTCTTTGGACTG 3'
CYCA3;1 For	5' AGGAAACCCTACAAGCAACAC 3'
CYCA3;1 Rev	5' CAGGGATGTTGTTTTGGAC 3'
CYCA3;2 For	5' CTGCTTCTGCTGTTTTTCTC 3'
CYCA3;2 Rev	5' TGGTAACATCCTCCCAAAAG 3'
CDKB1;1 For	5' ACTGGTGTTGACATGTGGTC 3'
CDKB1;1 Rev	5' CCCACTTAGGGTAAACATGC 3'
CDKB1;2 For	5' AGCTCCTGAAGTTTTGCTTG 3'
CDKB1;2 Rev	5' TGGATAGACATGCCAGTCAC 3'
CYCD1;1 For	5' CGGGTTCTTTATCTCCCATGC 3'
CYCD1;1 Rev	5' CAGTCTATAGCATCTCACTATCTTCTC 3'
CYCB1;4 For	5' ACAAAACCGGAAAGTTCTTG 3'
CYCB1;4 Rev	5' ATCACAGCATCCTTCAGTCC 3'
CYCB1;1 For	5' CGTCCTCGTACACGATCTC 3'
CYCB1;1 Rev	5' TTCTTCTCTAAACCACAAGCAG 3'
PARP1 For	5' ATCTTCTTGAGAGGCTTTGC 3'
PARP1 Rev	5' CCCTGACATTTGTCTGATTTAAG 3'
GAPC-2 For	5' CAAGGAGGAATCTGAAGGCAAAATGA 3'
GAPC-2 Rev	5' CAACCACACACAAACTCTCGCCG 3'

Table 1 - List of primers used in RT-PCR and qRT-PCR analysis

.into pDONR221 using BP clonase (Invitrogen). These entry clones were used in LR clonase (Invitrogen) reactions with pEarleyGate103 (Earley et al., 2006) to generate 35S::*RTE3-GFP* and 35S::*EER5-GFP*. Each construct was used to coat 1.0 μm gold particles, which were then transiently transformed into onion epidermal cells by bombardment with the Helios Gene Gun (Bio-Rad). After incubation in the dark for 24 hours at room temperature, epidermal peels were incubated in 5 μg/mL DAPI for 20 minutes. Peels were washed with water and imaged using a Zeiss AxioObserver.Z1 fluorescence microscope.

Conclusion

Contributions to plant science

For the past six years, I have worked at gaining insight into, generally, plant science, and more specifically, ethylene signaling. The intragenic suppressor, *etr1-11*, demonstrates a region within ETR1 that is important for maintaining ethylene receptor function. The knowledge gained about the *RTE3* gene and its encoded protein provides information about a new protein complex that is involved in seedling growth. The protein complex contains proteins previously identified in diverse processes such as ethylene and auxin response, and photomorphogenesis. That these proteins may be involved in a process as basic as seedling growth adds another level of importance to these proteins' functions.

The suppressors of *etr1-2*

In the past twenty years, screens for mutants that alter ethylene responses in *Arabidopsis* have been very effective in identifying components of the ethylene signaling pathway. The initial goal of this research was to discover new members of the ethylene signaling pathway through a forward genetics approach. More specifically, the approach was to study suppressors of *etr1-2* to identify genes that would not be found in the usual ethylene insensitivity or constitutive response screens. While the screen did yield the interesting mutants of *rte3* and *etr1-11*, it did not accomplish the goal of finding new members of the ethylene signaling pathway. It is possible to find new signaling pathway components with this screen, as evidenced by *RTE1* (Resnick et al., 2006). The problem is that one must be specific

with the phenotype one chooses and to perform additional tests with the mutants isolated in any screen.

While finding *etr1-11* did not accomplish the goal of finding a new component of the ethylene signaling pathway, it did provide insight into how the ETR1 protein works. The *rte3* mutant did not give any insight into the ethylene signaling pathway, however, as it was shown that *RTE3* does not have a role in ethylene signaling. This is one of the pitfalls of a mutagenic screen, as potential mutants must be screened and characterized for a specific phenotype. What appears to be a partial suppression mutant could turn out to have nothing to do with the studied pathway. That is why an ideal approach to any mutagenic screen would be to only look for a very specific phenotype, and discard all other mutants as not good enough.

In the past, the triple response has been a good tool for ethylene research, since it is a qualitative measure rather than a quantitative one. While other fields are forced to screen on a subjective 'strong' vs. 'weak' response, the triple response can be measured in terms of hypocotyl length (and root length) and subjected to statistical analysis. The *rte1* mutant was isolated based on the clear exhibition of a triple response phenotype. For *rte3*, the triple response phenotype was not so clear, and so the mutant obtained was not a true ethylene response mutant. The second ethylene phenotype of senescence turned out to be the test that revealed *rte3* as a non-ethylene phenotype, so in the future, screens for ethylene response mutants should be tested for both phenotypes before further characterization.

The *etr1-11* mutation gives insight into *ETR1* function

To understand the function of a gene, a series of loss-of-function mutants is usually required. Prior to this study, only two loss-of-function mutants had been isolated before, in a study that performed targeted mutagenesis of *ETR1* ethylene binding domain residues (Wang et al., 2006). In my study, I identified a third loss-of-function allele. The *etr1-11* mutation is the first missense loss-of-function mutation in any of the ethylene receptors that was isolated in a genetic screen. This residue, along with the loss-of-function mutations found in the study by Wang et al. (2006), define a region that is important for maintaining a functional ETR1 protein. However, these three residues are far outnumbered by residues that, when mutated, lead to ethylene insensitivity. This shows that there are many more residues important for maintaining ETR1 in an 'on' signaling state than for actually stabilizing a functional ETR1 protein.

This would seem a strange situation if the case were that the 'on' signal was a phosphorylation event or a transcriptional cascade. However, in the case of ETR1, the 'on' signal could be as simple as a protein interaction. If this were the case, it would make much more sense that many more residues are important for a protein interaction than for stabilizing ETR1. It has been hypothesized before that ETR1 transmits its 'on' signal simply by maintaining a protein interaction, possibly with CTR1 or RTE1. When ethylene binds, the receptor changes conformation to release the target protein, which allows responses to occur. The fact that there are very few residues important for stabilizing the ETR1 protein structure also indicates that the ETR1 protein lowest energy state is in the 'on' state, while releasing the target is an

energetically unfavorable one (Wang et al., 2006; Resnick et al., 2006). *etr1-11* may indicate a region for this interaction. If it does not indicate a region of interaction with a downstream target, it may define a region of interaction for intramolecular stability. Once a three-dimensional structure for ETR1 is solved, key interaction surfaces may emerge, and the *etr1-11* residue can be mapped to indicate how intra- and intermolecular interactions are affected.

Future directions in *ETR1* research

One possible question that can be addressed about the *etr1-11* is if it affects any ETR1-protein interactions. Since ETR1 is known to interact with CTR1, the interaction of ETR1 and CTR1 could be tested in the presence of the *etr1-11* mutation (Clark et al., 1998). The exact nature of the signal from ETR1 remains unknown. As mentioned above, the signal could simply be a protein-protein interaction, but this hypothesis remains unconfirmed. Also, the nature of the signal from ETR1 could be different than the signal from other ethylene receptors. Recent work in the lab has shown that *ETR1* is unique among ethylene receptors due to its interaction with *RTE1* (Resnick et al., 2006; Resnick et al., 2008; Rivarola et al, 2009). Therefore the question remains: Are the other ethylene receptors signaling in the same manner as ETR1? The story is complicated by the fact that the ethylene receptors can form higher order complexes, allowing for great diversity in the combinations and signaling patterns of the receptors (Gao et al., 2008). So, to answer this question, a variety of approaches will need to be taken, like biochemical assays to determine signaling activity, cell biological analysis to study protein localization or activity, and genetic approaches to analyze functional relationships.

Analysis of *RTE3* reveals a function for a novel protein complex

The *rte3* mutant was initially isolated as an ethylene response mutant, but was later identified as a seedling growth regulator. While not the original intent of the screen, the information gained through it is new and unique. Yeast two-hybrid analysis shows that RTE3, EER5, EIN2, and the CSN interact to form a multi-protein complex (Fig. 29). This could be one of the complexes of unknown function that CSN members are seen to participate in. These complexes, seen in gel filtration column fractions, are usually smaller than the CSN - a complex of around 450 kDa (Gusmaroli et al., 2007). The RTE3 protein itself is predicted to be 190 kDa by itself, so any protein complex containing RTE3 is likely to be a large one.

This protein complex appears to be localized to the nucleus, as four of the members have already been demonstrated to localize there. This would indicate a role in perhaps gene regulation or nuclear transport. The role this complex plays would most likely not be one the CSN traditionally plays, since none of the phenotypes typical *csn* mutants have are exhibited by *rte3* mutants. It is highly likely that *RTE3* plays a much more specific role, in only one or two processes within the cell, maybe as a regulator of CSN function, while the CSN itself is a much more broadly-acting complex.

Future directions in *RTE3* research

There are many questions that need to be answered about how the *RTE3* gene functions in regulating seedling growth. First, what is the precise function of RTE3 in a multi-peptide complex? Crystal structures of Sac3p in yeast have shown that Sac3p is a scaffold protein for several other proteins in the mRNA export complex

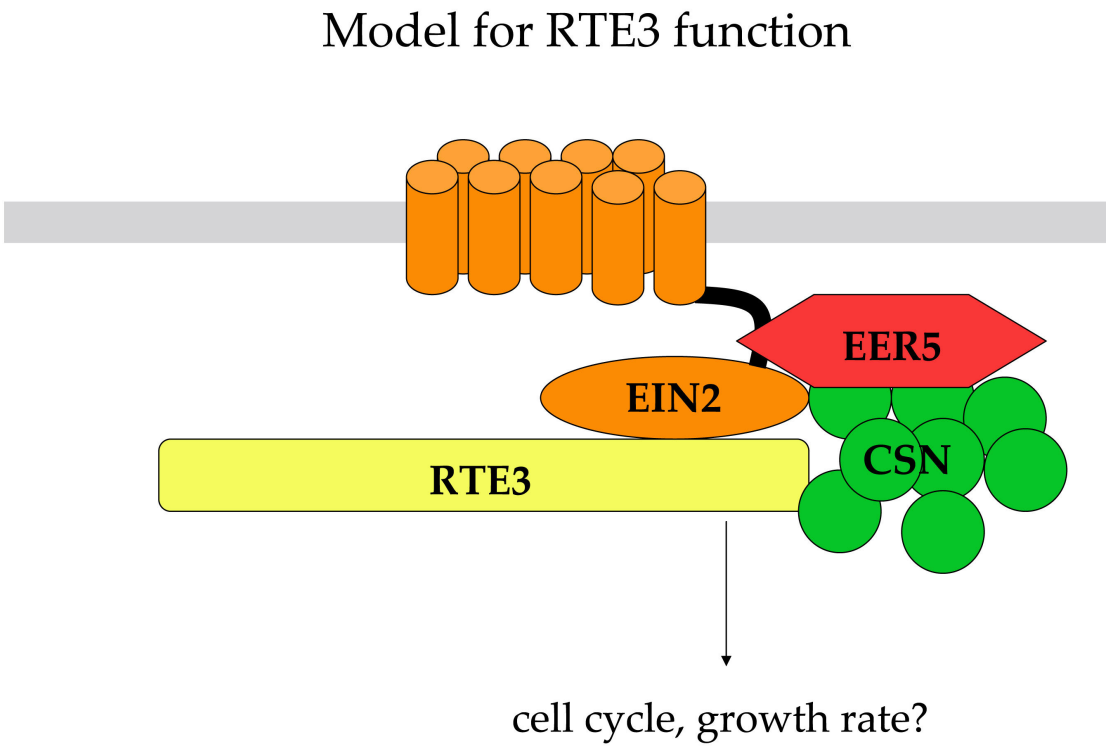


Figure 29 - Model for method of RTE3 function. RTE3 interacts with the C-terminus of EIN2, EER5, and a member of the CSN. These proteins may act together to regulate seedling growth, perhaps through control of the cell cycle. RTE3, EER5, and the CSN localize to the nucleus, so the localization of EIN2 may also be to the nucleus.

(Jani et al., 2009). This has not been verified in plants, although the yeast-two hybrid library screen result may indicate that some of the interactions remain the same. If the role of RTE3 is to act as a scaffold protein, are there any other proteins to be identified in the complex? This could be answered with a biochemical approach, by using a tagged RTE3 protein to pull down interacting proteins.

Another question to be answered about *RTE3* function is: What is the precise plant process that is being affected in *rte3* mutants? Right now the *rte3* mutant has been characterized as having defects in etiolated hypocotyl growth and root growth in the light. The nature of these defects remains unknown, however. There may be a slight defect in cell cycle regulation, but not as distinct as in *csn* mutants. Also, the effect could act on component downstream of the cell cycle, on elements that effect physiological changes. If RTE3 plays a minor role in cell cycle progression or control, it might be extremely difficult to carry out these studies in *Arabidopsis*, as synchronizing cells in this system is impossible. Other options exist, such as using cell cultures, or using techniques not dependent on synchronized cells. Another possibility is that cell expansion or tissue morphology could be affected, and these should be studied in greater detail, but there is currently no evidence to support the role of the CSN, EER5, or EIN2 in any of these processes.

Concluding Remarks

To end this dissertation, I reflect on the twists and turns a research project takes on the road to understanding. What one often sets out to accomplish, gets side-tracked on some detour or another. However, I feel new knowledge is gained through

the detours just as much as a straight line, and continue to hope that this is the case for the function of *RTE3* and *etr1-11* in the future.

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